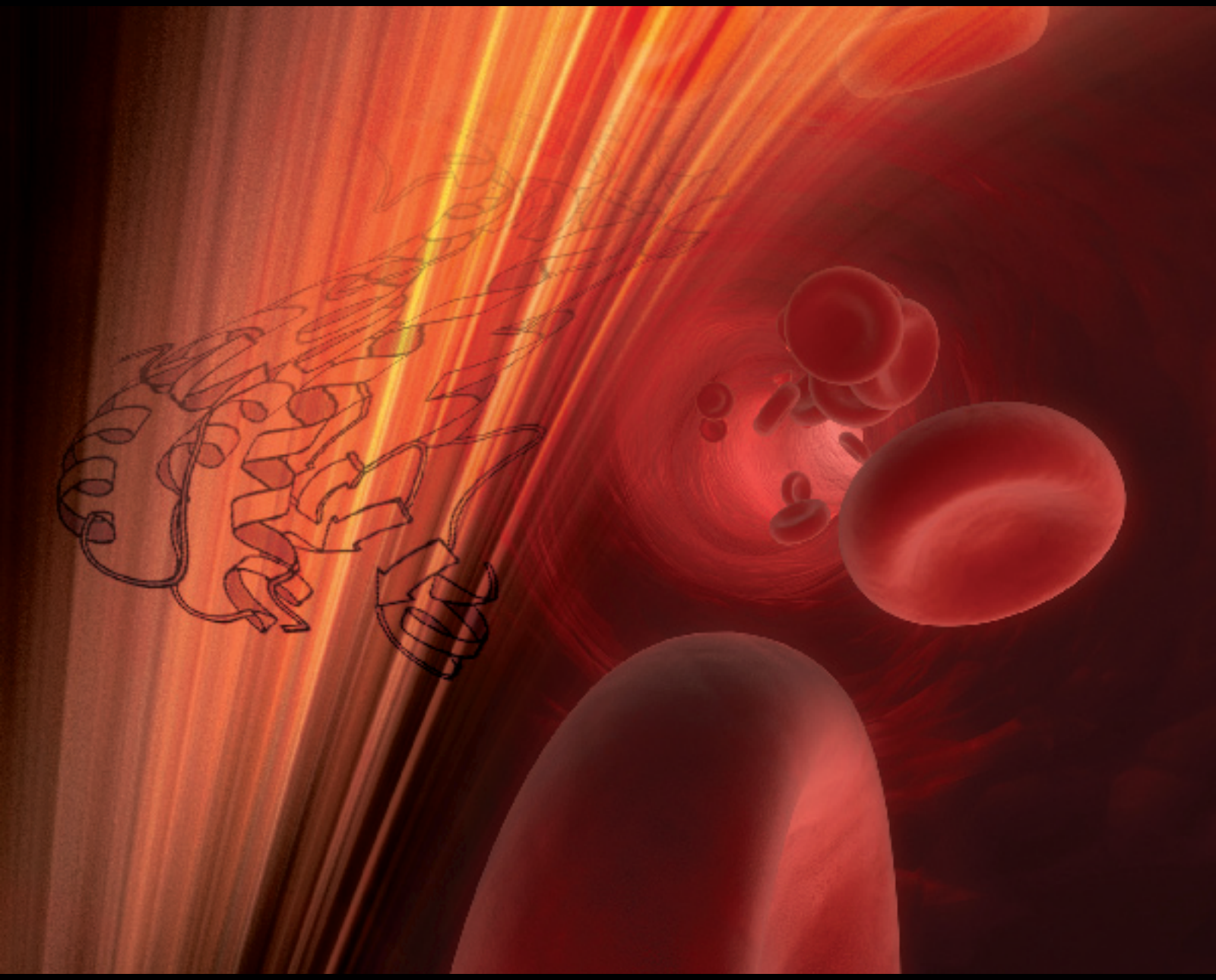
PPAR Research

PPARs and Xenobiotic-Induced Adverse Effects: Relevance to Human Health

Guest Editors: Christopher Lau, Barbara D. Abbott, J. Christopher Corton, and Michael L. Cunningham



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Editorial Board

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Editorial

PPARs and Xenobiotic-Induced Adverse Effects: Relevance to Human Health

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The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that act as transcription factors and play important roles in the regulation of a variety of biological processes, such as adipocyte proliferation and differentiation, glucose homeostasis, intracellular trafficking of lipids and their metabolism, inflammatory responses, vascular functions, and embryonic and fetal development. Three PPAR subtypes have been identified: PPARα, PPARβ/δ, and PPARγ (with isoforms PPARγ1 and PPARγ2), each with overlapping but unique ligand specificity, patterns of tissue distribution, and biological functions. The mechanisms of PPAR action have been well studied [1]. The nuclear receptors are activated by their ligands, heterodimerize with another nuclear receptor, retinoid X receptor (RXR), and undergo specific conformational changes that release corepressors and allow for recruitment of coactivators. The receptor complex binds to specific DNA sequences, called peroxisome proliferator response elements (PPREs), in the promoter regions of target genes for transactivation as well as transrepression. Activated genes are associated with fatty acid transport and metabolism, adipogenesis, peroxisome biogenesis, cholesterol and bile acid biosynthesis, proteasome activation, and glucose metabolism; repressed genes typically include those involved with adaptive inflammatory responses.

A number of endogenous ligands have been identified for each PPAR subtype and include long-chain polyunsaturated fatty acids such as linoleic and arachidonic acids, saturated fatty acids, and eicosanoids. Because of the involvement of PPARs in controlling energy homeostasis, synthetic chemicals have been designed to interact with these nuclear receptors for therapeutic intervention of a number of metabolic diseases such as obesity, type-2 diabetes, and atherosclerosis. Indeed, in the past several decades, specific pharmacologic agents such as the fibrates (that include clofibrate, fenofibrate, ciprofibrate, bezafibrate, and gemfibrozil) and the glitazones (such as ciglitazone, troglitazone, rosiglitazone and pioglitazone) have been developed that target PPARα and PPARγ, respectively, for the effective treatment of hyperlipidemia and diabetes. Since 1990 when the PPAR family members were cloned and characterized, a number of industrial and consumer chemicals, pesticides, and environmental contaminants have been shown to activate PPARs. These include di-(2-ethylhexyl)phthalate (DEPH) [2], diisobutyl phthalate [3], trichloroethylene, di- and trichloroacetic acids, [4], bisphenol A [5], butylparaben [3], perfluoroalkyl acids (PFAAs) [6], and organotins [7]. Systematic screening of chemicals in commerce and in the environment for PPAR molecular signature and functional activities may further expand the existing list [8–10]. However, the potential human and ecological health risks from such chemically induced PPAR activation are still relatively unknown and presently subject to great debate.

This special issue is organized to highlight the recent advances made in identifying drugs and chemicals that target PPARs as their mechanism-of-action, in characterizing

the downstream biochemical and physiological consequences from these drug actions and chemical insults, and in addressing the relevance of this mechanism-of-action and toxicity for human health risks. This issue will focus on both cancer and noncancer effects (that include reproductive, developmental, immunologic and metabolic endpoints), and unique actions mediated by the different PPAR subtypes.

There are eight papers in this special issue, including five original research articles and three reviews. In the first research article, "*Peroxisome proliferator-activated receptors alpha, beta, and gamma mRNA and protein expression human fetal tissues*," B. D. Abbott et al. characterize the mRNA and protein expression of the three PPAR subtypes in human fetal tissues. With the exception of one study that previously described the expression of PPAR proteins in the human fetal digestive tract, this is the first comprehensive report to compare the expression of these nuclear receptor subtypes in human fetal liver, heart, lung, kidney, stomach, intestine, adrenal, spleen and thymus during organogenesis, and to contrast the levels of expression in the fetus to those in adult tissues. This study reports that PPARα, β and γ were expressed in all nine human fetal tissues evaluated. In general, mRNA expression of PPAR subtypes varied by tissue; notably, the levels in fetus were comparable to or even higher than those in adult, a pattern similar to that observed in rodents. These findings indicate that PPARs likely serve key roles in regulating developmental events, and inappropriate or untimely activation of these nuclear receptors (through transplacental delivery of drugs or exposure to xenobiotic chemicals) may bear untoward health consequences. Subsequent to the appearance of this paper online, these investigators discovered an artifact in their detection for PPARγ proteins, and have conducted an additional study to rectify the unexpected error. The replacement findings have now been published in "*Erratum to "Peroxisome proliferator activated receptors alpha, beta, and gamma mRNA and protein expression in human fetal tissues,*"" and the new protein results are in good agreement with the patterns of expression obtained for PPARγ mRNA in these tissues.

In a review article entitled "*The role of PPARα activation in liver and muscle*," L. Burri et al. summarize the involvement of PPARα in two metabolically active tissues, liver and skeletal muscle, and provide a comparative overview of the benefits and risks of PPARα activation in humans and rodents. The beneficial effects of PPARα activation in counteracting metabolic disorders are well supported in both animal and human studies. Indeed, both species share multiple changes in expression of genes that belong to functional classes related to lipid metabolism. Yet, there are substantial differences between human and mouse target gene expression in response to PPARα activation in the liver, particularly those associated with peroxisome proliferation, hypertrophy, hyperplasia, apoptosis and tumor induction. The responses to PPARα activation appear to be more pronounced in mice than in humans. In contrast to mice, humans show no effect on glucose metabolism in response to PPARα activation; conversely, apolipoprotein production that leads to a decrease of VLDL and an increase of HDL cholesterol is only seen in humans treated with a PPARα activator. PPARs are expressed in skeletal muscles in humans and rats; activation of these nuclear receptors increases lipid oxidation and decreases triglyceride accumulation and alters glucose metabolism. These investigators note a sex difference in both humans and rodents in response to PPARα activation and caution that gender differences should be taken into consideration for therapy involving PPARs.

The theme of sex differences related to PPARα effects is continued in a second review article presented by M. Yoon "*PPARα in obesity: sex difference and estrogen involvement*" who describes sexual dimorphism in the treatment of obesity by PPARα ligands and summarizes the involvement of estrogen. Both PPARα and estrogen receptors (ERs) are involved in regulating adiposity. Interestingly, PPAR/RXR heterodimers have been shown to bind to estrogen response elements, and PPARs and ERs share certain cofactors, suggesting that signal cross-talk between these two nuclear receptors may participate in the control of obesity. However, sex-related differences have been reported in PPARα effects in animal studies. Fenofibrate reduced weight gain and adiposity in male mice given a high-fat diet and reduced circulating cholesterol and triglycerides, while females exhibit drug resistance. In fact, estrogens appear to inhibit PPARα action on obesity. While both fenofibrate and estradiol (E2) by themselves were effective in attenuating weight gain and increases of fat mass in mice fed a high-fat diet, combined fenofibrate and E2 treatment did not produce any additional effects; the combined treatment actually led to elevated levels of circulating cholesterol and triglycerides compared to those with each treatment alone. Findings from animal studies are by and large in agreement with clinical observations. Details about the interplay between PPARα and ERs are presently unavailable, but competition between these two nuclear receptors for transcriptional coactivators and corepressors may confer a negative cross-talk between their actions.

A research article by M. Cunningham et al. "*Effects of the PPARα agonist and widely used antihyperlipidemic drug gemfibrozil on hepatic toxicity and lipid metabolism*" follows the discussion of the use of antihyperlipidemic drugs, focusing on lipid metabolism and hepatic toxicity of another fibrate, gemfibrozil, and comparing the responses between rats, mice, and hamsters. Gemfibrozil is a valuable therapeutic agent in the control of coronary heart disease, in part due to its hypolipidemic effects in reducing levels of triglycerides and LDL cholesterol and raising those of HDL. Similar to other peroxisome proliferators, gemfibrozil is known to induce liver hypertrophy and tumors in rodents. This paper summarizes the results of several studies conducted by the National Toxicology Program to evaluate the effects of chronic exposure to gemfibrozil in rats and mice; evaluation of hamsters is included because this species, like humans, is relatively resistant to the hepatotoxicity and carcinogenicity of peroxisome proliferators. In general, hepatic effects of gemfibrozil were seen in all three species, although rats appeared to be most responsive and hamsters to be least responsive. Correspondingly, a similar rank order of species difference was noted in the oxidative stress-related mechanisms-of-action produced by gemfibrozil, which may be related to the differential susceptibility to the hepatocarcinogenicity of

this drug. Information provided in this paper should lend support in differentiating the beneficial effects of PPARα drugs in treating dyslipidemia and their potential risks of tumor induction.

Two research articles by C. J. Wolf et al., "*Developmental effects of perfluorononanoic acid in the mouse are dependent on peroxisome proliferator-activated receptor-alpha*," and M. B. Rosen et al., "*Gene expression profiling in wild-type and PPARα-null mice exposed to perfluorooctane sulfonate reveals PPARα-independent effects*," address the potential human health risks of perfluoroalkyl acids, a class of persistent environmental contaminants that has received intense scrutiny from regulatory agencies worldwide. PFAAs are found ubiquitously in all environmental media, distributed globally, present in humans and wildlife, and associated with several adverse effects in laboratory animal models. These chemicals vary in carbon-chain lengths and functional groups (chiefly carboxylates and sulfonates), but all appear to activate mouse and human PPARα [6]. PPARα activation by PFAAs has been shown previously to be related to their hepatotoxicity, developmental toxicity, and immunotoxicity in rodents. Results from previous studies with transgenic PPARα-null mice have indicated that developmental toxicity of perfluorooctanoate (PFOA), but not that of perfluororooctane sulfonate (PFOS), is dependent on PPARα [11, 12]. Using a similar experimental design, C. J. Wolf et al. in "*Developmental effects of perfluorononanoic acid in the mouse are dependent on peroxisome proliferator-activated receptor-alpha*" report that the adverse developmental effects of perfluorononanoic acid (PFNA) were more pronounced than those of PFOA, but also dependent on a PPARα mechanism. Thus, neonatal mortality (at high doses), growth impairment and developmental delays (at lower doses) were observed in wild-type mice but not in PPARα-null mice after gestational exposure to PFNA. These results therefore confirm a different mode-of-action for developmental effects between the perfluoroalkyl carboxylates and perfluoroalkyl sulfonates, and that the chemical potency of PFAAs increases with carbon-chain length. In contrast, the phenotypic responses in the liver of mice exposed to PFOA or PFOS are quite similar. Both fluorochemicals activate PPARα and its target genes, inducing peroxisome proliferation, hypertrophy and tumors in the liver. However, M. B. Rosen and coworkers in "*Gene expression profiling in wild-type and PPARα-null mice exposed to perfluorooctane sulfonate reveals PPARα-independent effects*" report a number of genomic changes associated with lipid metabolism, inflammation and xenobiotic metabolism that are independent of PPARα activation; rather, these gene expressions may be related to PPARγ, PPARβ, or another nuclear receptor, the constitutive androstane receptor (CAR), thus indicating the possibility of multiple modes-of-action for PFAA hepatic effects. In addition, altered expression of certain genes unique to PFOS exposure was identified, including those associated with ribosome biogenesis, oxidative phosphorylation and cholesterol biosynthesis. These findings should provide valuable support for the assessment of human health risks of exposure to these environmental contaminants.

Continuing the exploration of target genes activated by PPARα and their attendant functional responses, H. Ren et al. in "*Regulation of proteome maintenance gene expression by activators of peroxisome proliferator-activated receptorα*" focus on the regulation of proteome maintenance (PM) by this nuclear receptor. Increased oxidative stress caused by chemical or physical insult can lead to misfolding or other damage to protein, and restoration of cellular homeostasis entails stabilization of unfolded proteins by molecular chaperones (such as heat shock proteins, Hsp) or removal of damaged proteins by proteolysis. Ample evidence has suggested that PPARα protects multiple tissues from oxidative stress induced by chemicals through altered expression of genes involved in proteome maintenance, including those in the Hsp family and proteasomal genes involved in proteolysis. These investigators compare and contrast the expression of PM genes with traditional target genes (e.g., lipid metabolizing enzymes) in rodent liver after exposure to seven diverse peroxisome proliferators (WY 14,643, fibrates, valproic acid, DEHP, and PFAAs). Genes and proteins involved in proteome maintenance were altered by these peroxisome proliferators, although the expression of many of these genes appeared to be delayed or transient, and was distinctly different from other typical PPARα-dependent genes. These results therefore support an expanded role for PPARα in regulating genes and proteins that serve as guardians of the proteome, in addition to controlling lipid metabolism and energy balance.

A. Rogue et al. in "*Gene expression changes induced by PPAR gamma agonists in animal and human liver*," summarize the changes of hepatic gene expression induced by PPARγ agonists in animal models and humans. PPARγ is highly expressed in adipose tissues, and to a much lesser extent in the liver. PPARγ drugs such as the glitazones are used to treat type-2 diabetes. They enhance insulin sensitivity presumably by channeling circulating fatty acids into adipose tissue. However, side effects of at least one of these agents include idiosyncratic hepatotoxicity, although the determinant factors for the untoward actions of PPARγ agonists remain to be elucidated. The authors compare the gene expression profiles of PPARγ activation derived from *in vivo* studies with rodent livers to those obtained from *in vitro* studies with rat and human hepatocytes. PPARγ levels are enhanced in obese and diabetic mouse liver, and the steatogenic responses to glitazone in these rodent models are more pronounced than those seen in the lean controls. The genomic responses to PPARγ agonists in the liver mirror the tissue distribution profile of this nuclear receptor; hence, only a small number of genes were affected in the liver compared to the adipose tissues. Only limited studies are available with human liver cells, and results from individual donors are quite variable, perhaps in line with the idiosyncratic nature of the hepatotoxicity observed. Future studies identifying specific PPARγ genes in the liver will elucidate the etiology of hepatotoxicity associated with PPARγ agonists, particularly after long-term therapeutic treatment.

In summary, this special issue provides a glimpse of the current understanding of PPAR involvement in therapeutic

interventions, as well as the untoward side effects, and the potential health risks from exposure to xenobiotic chemicals found in the environment. These reviews and research papers contribute significantly to our understanding of these intriguing nuclear receptor signaling molecules.

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Research Article

Peroxisome Proliferator-Activated Receptors Alpha , Beta, and Gamma mRNA and Protein Expression in Human Fetal Tissues

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Peroxisome proliferator-activated receptors (PPARs) regulate lipid and glucose homeostasis, are targets of pharmaceuticals, and are also activated by environmental contaminants. Almost nothing is known about expression of PPARs during human fetal development. This study examines expression of PPARα, β, and γ mRNA and protein in human fetal tissues. With increasing fetal age, mRNA expression of PPARα and β increased in liver, but PPARβ decreased in heart and intestine, and PPARγ decreased in adrenal. Adult and fetal mean expression of PPARα, β, and γ mRNA did not differ in intestine, but expression was lower in fetal stomach and heart. PPARα and β mRNA in kidney and spleen, and PPARγ mRNA in lung and adrenal were lower in fetal versus adult. PPARγ in liver and PPARβ mRNA in thymus were higher in fetal versus adult. PPARα protein increased with fetal age in intestine and decreased in lung, kidney, and adrenal. PPARβ protein in adrenal and PPARγ in kidney decreased with fetal age. This study provides new information on expression of PPAR subtypes during human development and will be important in evaluating the potential for the developing human to respond to PPAR environmental or pharmaceutical agonists.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily and there are three primary subtypes (α, β/δ, and γ) [1]. These receptors play important roles in embryonic and fetal development as well as placental function [2, 3], regulating many cellular and metabolic processes [4]. PPARs control energy homeostasis, are important regulators of adipogenesis, lipid metabolism, inflammatory responses, and hematopoiesis, and are implicated in chronic diseases such as diabetes, obesity and atherosclerosis [5–8]. PPARβ and γ have roles in early embryonic survival and implantation [9, 10]. PPARs regulate gene expression by binding to specific DNA sequences, peroxisome proliferator response elements (PPREs), in the promoter regions of target genes. Prior to DNA binding PPAR forms a heterodimer with the retinoid X receptor (RXR) [11, 12]. A number of endogenous ligands have been identified for each PPAR subtype, including long-chain fatty acids, polyunsaturated fatty acids such as linoleic and arachidonic acids, saturated fatty acids, and eicosanoids [1]. A variety of synthetic ligands have been developed for pharmaceutical purposes to treat chronic diseases such as hyperlipidemia, diabetes, and metabolic syndrome. In addition, some chemicals and environmental contaminants activate PPARs, for example, phthalates, tri- and dichloroacetic acids, trichloroethylene, and the perfluorinated alkyl and sulfonyl acid compounds (PFAAs) [11–14].

PFAAs, including perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorooctane sulfonate (PFOS), are highly stable molecules with chemical properties that make them excellent surfactants [15]. For many years these chemicals were widely used in industrial applications and are now found as persistent environmental contaminants that are also present in the tissues and serum of wildlife and humans [15–17]. In laboratory studies, prenatal

exposure of rodents to these compounds produces dose-related effects on pre- and postnatal survival, developmental delay, and deficits in postnatal growth [18–23]. PFAAs activate PPARα, and the developmental toxicity of PFOA and PFNA in the mouse was shown to be dependent on expression of PPARα in the fetus (effects of prenatal exposure to PFOA or PFNA that occur in wild-type mice were not observed in PPARα knockout offspring) [24, 25]. PPARs are expressed in the mouse embryo and fetus (reviewed in [26]), and prenatal exposure to PFAAs was shown to change gene expression in the pre- and postnatal livers in a pattern indicative of activation of PPAR as well as the CAR nuclear receptor [27–29].

Almost nothing is known about expression of PPAR during human development. At the present time, a search of the literature revealed only one paper that described the expression of PPARs in the human fetus, and that paper described expression in the gastrointestinal (GI) tract [30]. This gap in scientific knowledge of PPAR expression during human development requires attention as PFAAs, which activate PPAR, alter gene expression, and have developmental toxicity in the rodent, are pervasive in the environment and have been found in serum and blood samples of populations around the world, including samples from infants, children, and in umbilical cord blood and milk (indicating prenatal and postnatal exposure of infants) [17, 31–36]. Thus, in order to evaluate the potential for these environmental compounds, and others, to affect human fetal development, it is important to have information regarding the expression of PPARs in the developing human fetus. The present study reports mRNA and protein expression for PPARα, β, and γ in embryonic day (ED) 54 to 125 human fetal liver, heart, lung, kidney, stomach, intestine, adrenal, spleen, and thymus.

2. Methods and Materials

2.1. Human Fetal and Adult Samples. Human fetal tissues ranging in age from embryonic day (ED) 54 to 125 were obtained from the Birth Defects Research Laboratory at the University of Washington, Seattle. The collection of tissue specimens from clinically aborted fetuses by the Birth Defects Research Laboratory (including informed consent for the donation and all procedures) was conducted with Human Subjects Institutional Review Board (IRB) approval. At the EPA, the study was reviewed by the Office of Human Research Ethics, UNC Biomedical IRB, and approved by the National Health Effects and Environmental Research Laboratory (NHEERL) Human Research Protocol Office (HRPO).

Tissues were snap frozen as soon as possible after collection and stored at −80°C until shipped on dry ice. On arrival at EPA, samples were stored at −80°C until processed for total RNA and protein. The nine tissues analyzed included liver, heart, lung, kidney, stomach, intestine, adrenal, spleen, and thymus. Prior to processing the fetal tissues to prepare RNA and protein, samples were weighed and smaller samples were designated for RNA preparation only, while larger samples were divided for both RNA and protein preparation, and any excess sample was returned (still frozen) for storage at −80°C. Handling during the weighing and division of samples was done over dry ice to the extent possible to minimize thawing. Adult total RNA for the 9 tissues examined in the study was obtained from FirstChoice Human Total RNA Survey Panel, Ambion, Inc, (each adult sample consisted of pooled total RNA from 3 individuals). In addition, tissue samples from 23 adult human livers were available for comparison of PPAR mRNA expression in adult and fetal liver. These samples were obtained from CellzDirect, Inc. (Durham, NC). Total RNA was prepared from the frozen adult liver tissue samples and qPCR performed, as described for the fetal samples.

2.2. qPCR Experimental Design and Procedures. Each tissue was run in separate qPCR experiments (e.g., liver samples were not run with those of any other tissue). In the qPCR experiments, expression of PPARα, β, γ and an internal control gene were examined on each plate, and samples on the plate included 2 replicates of each fetal sample and of the appropriate pooled adult tissue (FirstChoice Human Total RNA). In cases where there were too many samples of a tissue to run all of the reactions on one plate, the samples were run across 2 plates such that each age was represented as equally as possible on each plate. The actual number of samples examined for each tissue is stated in the results section, but the number available ranged from 23 to 46 specimens, except for thymus which had 11 specimens. PPAR gene expression was expressed relative to an internal control gene. The fetal samples of each tissue were examined for expression of β-actin, β2-microglobulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as potential internal control genes. Regression analysis of cycle threshold (Ct) was performed for each potential control gene to detect any changes in expression with age. Based on favorable regression outcomes in all of the tissues (no significant change with age), β2-microglobulin (B2M) was selected as the internal control gene (data not shown).

Tissue was homogenized and extracted in TRI Reagent (Sigma Chemical, St. Louis, MO) according to the manufacturer's directions, and RNA pellets were stored in 70% ethanol at −80°C until further use. Following resuspension in nuclease-free water (Promega Corporation, Madison, WI), the RNA was quantified and evaluated for purity (260 nm/280 nm and 260 nm/230 nm ratio) using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,Wilmington, DE). Prior to qPCR, 2 μg total RNA was digested using 2 units of DNaseI (Promega Corporation, Madison, WI) for 30 min at 37°C followed by 10 min at 65°C in a buffer containing 40 mM Tris (pH 8.0), 10 mM $MgSO_4$, and 1 mM $CaCl_2$. The DNase-treated RNA was then quantified using a Quant-iT RiboGreen RNA assay kit according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA). Approximately 1 μg of the DNase-treated RNA was reverse transcribed using a High-Capacity cDNA Archive Kit according to the provided protocol (Applied Biosystems). Amplification was performed on an Applied Biosystems model 7900HT Fast Real-Time PCR System in duplicate using 25 ng cDNA and TaqMan Universal

PCR Master Mix (Applied Biosystems) in a total volume of 12 μl. The following TaqMan assays (Applied Biosystems) were included in the study: PPARα (Hs00947539_m1), PPARβ (Hs00602622_m1), PPARγ (Hs00234592_m1), β-actin (Hs99999903_m1), GAPDH (Hs99999905_m1), β-2 microglobulin (Hs99999907_m1).

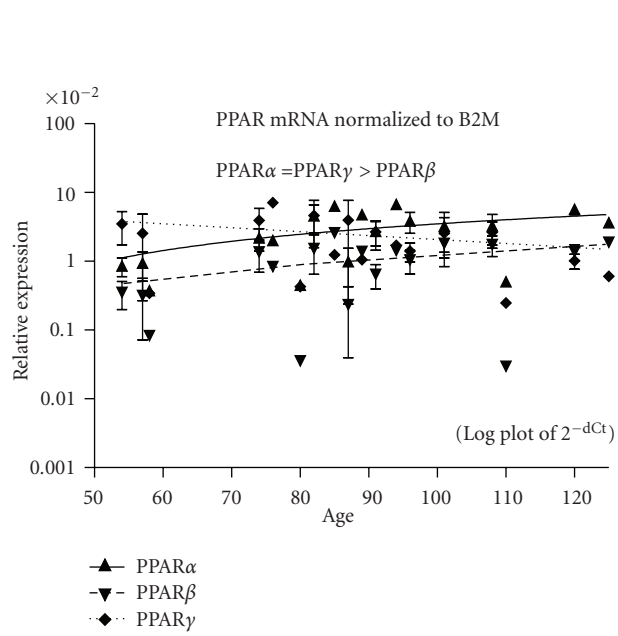
PPAR mRNA Ct values, calculated by Applied Biosystems SDS2.2.2 software, were normalized by subtraction of the Ct for the internal control, B2M, generating δCt values. The mean δCt for each sample was calculated from the 2 replicates and then analyzed to evaluate changes in expression with fetal age (regression analysis). Differences in expression between subtypes were determined using ANOVA of all mean δCt values (without regard to age), with Bonferroni's post-test applied for pairwise comparisons (Prism 4.0, GraphPad Software, San Diego, CA). Data are graphed as a log plot of $2^{-\delta Ct}$. For all tissues except liver, a comparison of the fetal samples with the single adult pooled sample was performed using Ct values and a t-distribution test (sample size less than 30) or the Z-distribution test (sample size equal to or greater than 30) to determine the probability that an adult value of this extreme or more extreme would be found in the distribution of fetal values (using probability calculators available on-line at http://faculty.vassar.edu/lowry/tabs.html or http://davidmlane.com/hyperstat/z_table.html). As the internal control gene expression was not the same in adult and fetal tissues, Ct values, and not δCt values, were used for this comparison. Data comparing fetal (mean Ct of 2 replicates) and adult Ct (each replicate shown in the plot) are graphed as a vertical scatter plot of Ct. For the liver, there were 23 adult liver specimens available for comparison with the fetal liver samples. The adult samples were from both males and females and ranged in age from 21 to 86 years, but analysis of either Ct or dCt showed no effect of either sex or age on the expression of PPARα, β, or γ or B2M (data not shown). The adult and fetal liver samples had comparable levels of the internal control gene, B2M, and thus it was possible to compare the normalized Ct values (dCt) using ANOVA with Bonferroni's post-test applied for pairwise comparisons (Prism 4.0, GraphPad Software, San Diego, CA).

2.3. Western Blot Experimental Design and Procedures. Samples of each tissue were run in separate Western blot experiments (e.g., liver samples were not run with those of any other tissue). In general, most of the tissues required 2-3 Western blots to accommodate all of the samples (only 12 sample lanes were available per gel), and the samples were blocked across blots such that the age range was represented as uniformly as possible on each blot. The actual number of samples examined for each tissue is stated in the results section, but the number available ranged from 5 to 36 specimens, and only thymus and spleen had fewer than 22 specimens. Each blot was examined for expression of one of the PPAR subtypes and for the internal control protein. GAPDH was selected as the internal control protein as expression did not change with age (based on regression analyses of GAPDH protein expression across age in each of the 9 tissues, data not shown). A positive control for antibody detection of PPARα, β, or γ was also run on each blot. Positive controls were Hep G2 whole cell extract (Santa Cruz, SC-2227), Jurkat cell nuclear extract (Santa Cruz, SC-2132), and U937 whole cell extract (Santa Cruz, SC-2239), for expression of PPARα, β, or γ, respectively. After all tissues were examined in this manner, additional Western blots were run in which all 9 tissues were represented on the blot, with most of the tissues from the same 91-day-old fetus (to the extent possible, as not all tissues were available from any single fetus, and thymus was not available from a 91-day fetus). Three "across-tissue" blots were run for each PPAR subtype (i.e., $n = 3$ fetuses per tissue examined in the "across-tissue" survey).

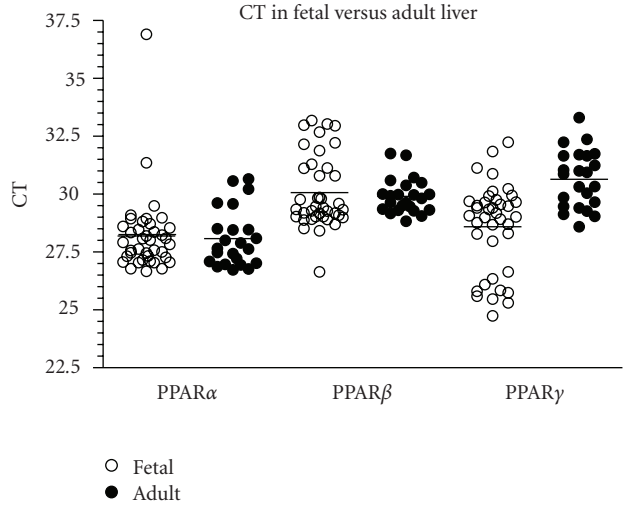
Western blots were run with 25 μg of total cell lysate or positive control per lane. All gels were 7.5% acrylamide precast gels (Biorad, Hercules, CA) and were run for 90 min at 125 V. Protein transfer to nitrocellulose membrane (Biorad) was done for 40 min using the Biorad semidry transfer system. Membranes were blocked for 1 hr in 5% milk and incubated overnight in primary antibody in 5% milk. Primary antibodies for PPARα (SC-9000) and PPARγ (SC-7196) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA) and used at a dilution of 1:200. PPARβ (Abcam 21209) antibody was obtained from Abcam, Inc, (Cambridge, MA) and used at 1 : 750 dilution. Antibody for GAPDH (SC-25778) was from Santa Cruz Biotechnologies and was diluted at 1 : 10,000. After overnight incubation with primary antibody, blots were probed 1 hr with a horseradish peroxidase-conjugated secondary antibody in 5% milk. Secondary antibodies were diluted 1 : 5000 and included goat anti-rabbit Jax 111-035-144 (West Grove, PA), goat anti-rabbit KPL 074-1506 and rabbit anti-goat KPL 14-13-06 (KPL, Gaithersburg, MD). Chemiluminescence was imaged using a Biorad Fluor-S machine with 2 or 5 min exposures. Biorad Quantity One software was used to perform volume rectification densitometry with background subtraction on the chemiluminescence images, generating data for both the PPAR and GAPDH bands. PPAR protein expression was expressed relative to the internal control gene, GAPDH, and regression analysis of the relative values was performed to detect any significant change in slope with age (Prism 4.0, GraphPad Software, San Diego, CA).

3. Results

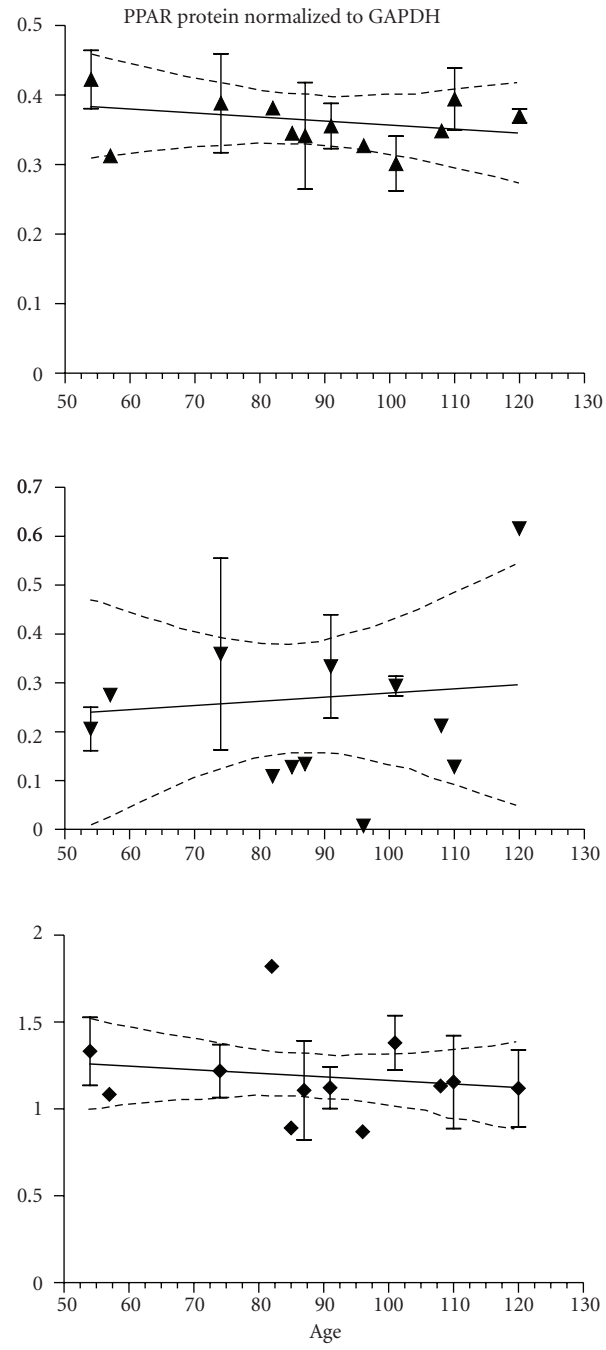
The expression of PPARs is presented for each tissue, reporting any change in expression of protein or mRNA with gestational age, comparing the relative level of mRNA expression of the isotypes, and comparing mRNA expression in the fetal organ to that observed in the human adult sample of that tissue. Protein and mRNA for all PPAR isotypes were detected in all of the 9 tissues and the results are summarized in Table 1, which also lists the tissues from highest to lowest expression of mRNA for each PPAR. Table 2 summarizes the relative expression of the isotypes within each tissue. The data is shown using the same presentation format for each tissue. A full narrative presentation is given for the first tissue presented (liver) and, for the sake of brevity, the results for



(a)



(b)



(c)

Figure 1: Liver. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult liver. Each symbol represents the mean Ct value of 2 replicates for each fetal (open circles) and adult (filled circles) sample (overall mean for each group is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density is normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

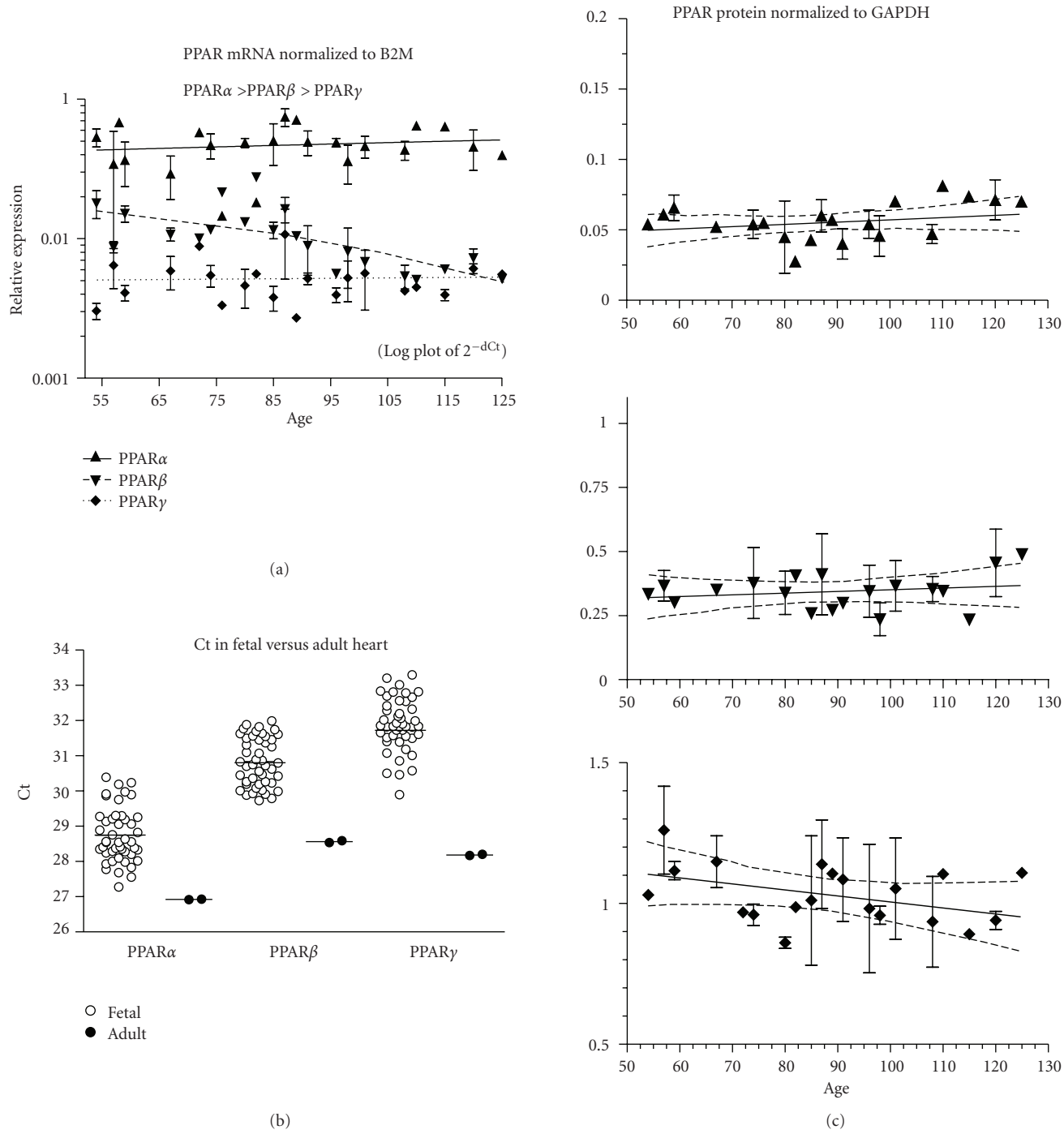


Figure 2: Heart. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult heart. Each symbol represents the mean Ct value of 2 replicates for each fetal (open circles) sample and adult (filled circles) individual replicates are shown (overall mean for each group is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

other tissues omit repetitive explanations which apply to all the data sets.

3.1. Liver. Human fetal liver expressed PPARα, β, and γ mRNA from ED54–125 (data acquired from 39 fetuses). Expression of PPARα and PPARβ increased significantly while PPARγ remained unchanged across the age range (Figure 1(a), $P < .01$ and .05, resp.). Expression levels of PPARα and γ across all ages were not significantly different and both were more highly expressed than PPARβ ($P < .001$). β2M mRNA expression was considerably higher than any of the PPAR subtypes (mean ± SEM Ct across all ages, note that a lower Ct signifies more abundant mRNA than a high Ct: β2M = 22.4 ± 0.3, PPARα = 28.2 ± 0.3, PPARβ = 30.1 ± 0.3, PPARγ = 28.6 ± 0.3). The expression of PPAR mRNA in the fetal liver was compared to that in 23 adult human liver samples. The adult samples were from both males and females and ranged in age from 21 to 86 years, but analysis showed no effect of either sex or age on the expression of PPARα, β, γ, or B2M (data not shown). The expression of B2M, the internal control gene, in adult and fetal liver samples was not significantly different (21.9 ± 0.2, 22.4 ± 0.3, mean ± SEM, resp.), and thus it was possible to analyze PPAR values normalized to B2M (dCt). Analysis of unadjusted Ct or dCt gave the same outcomes, and Figure 1(b) shows plots of the Ct values for adult and fetal livers. The human fetal and adult livers expressed PPARα and β mRNA at levels that were not significantly different (Figure 1(b)), but PPARγ was significantly higher in the fetal liver ($P < .001$, lower mean Ct indicates higher mRNA present in a sample). The overall outcome for PPARα and β was the same from the pooled total RNA from 3 donors (Ambion FirstChoice liver sample, data not shown) as that from the 23 individuals; adult and fetal liver expression did not significantly differ. The Ambion FirstChoice pooled adult liver RNA indicated that PPARγ did not differ between adult and fetal livers, but the data from the 23 individuals showed a significantly higher expression in the fetus, and the larger "n" of that assay would lend support to the validity of that outcome. In the fetal liver, PPARα, β, and γ protein expression did not change with fetal age (Figure 1(c); data from 22 fetuses, ED54–120).

3.2. Heart. PPARα and γ expression did not change, but PPARβ expression decreased ($P < .0001$) with fetal age (Figure 2(a), ED54–125, $n = 46$ fetuses). PPARα, PPARβ, and PPARγ relative expressions are shown in Table 2 and differences between isotypes were significant at $P < .001$. β2M mRNA expression was higher than PPAR (mean Ct ± SEM: β2M = 24.2 ± 0.8, PPARα = 28.7 ± 0.1, PPARβ = 30.8 ± 0.1, and PPARγ = 31.9 ± 0.1). Fetal PPARα, β, and γ mRNA expression was lower than that of the adult sample (Figure 2(b); $P < .05$,.001,.001, resp.). PPAR protein expression did not change with fetal age (Figure 2(c); 36 fetuses, ED54–125).

3.3. Lung. PPAR mRNA expression in fetal lung did not change with age (Figure 3(a), ED54–120, $n = 27$ fetuses). PPARα was the most highly expressed isotype ($P < .001$, Table 2). β2M mRNA expression was higher than PPAR, (mean Ct ± SEM: β2M = 24.0 ± 0.2, PPARα = 28.4 ± 0.1, PPARβ = 29.4 ± 0.1, and PPARγ = 29.5 ± 0.2). Fetal and adult PPARα and β mRNA expressions were not different, but fetal PPARγ was lower (Figure 3(b); $P < .05$). PPARα protein levels decreased ($P < .05$), but PPARβ and γ did not change with fetal age (Figure 3(c), ED57 to 120, $n = 27$ fetuses).

3.4. Kidney. PPAR mRNA expression did not change with age (Figure 4(a), 46 fetuses, ED54–125). PPARγ expression was higher than PPARα and PPARβ ($P < .05$, $P < .001$, resp.), and PPARα was higher than PPARβ ($P < .001$). β2M mRNA expression was higher than PPAR (mean Ct ± SEM: β2M = 24.5 ± 0.1, PPARα = 29.3 ± 0.1, PPARβ = 30.0 ± 0.1, PPARγ = 28.9 ± 0.1). PPARα and β fetal mRNAs were lower than in the adult (Figure 4(b); $P < .01$, <.0001, respectively), but PPARγ was similar ($P = .07$). PPARβ protein expression did not change with fetal age ($P = .09$), but PPARα and γ decreased ($P < .05$, Figure 4(c); 36 fetuses, ED57–125).

3.5. Stomach. PPAR mRNA expression did not change with age (Figure 5(a), 35 fetuses, ED54–120). PPARγ was the most highly expressed isotype ($P < .001$, Table 2). β2M mRNA expression was higher than PPAR (mean Ct ± SEM: β2M = 28.0 ± 0.3, PPARα = 32.4 ± 0.3, PPARβ = 33.3 ± 0.3, and PPARγ = 29.8 ± 0.4). PPARα, β, and γ mRNA expression was lower in fetal than in adult stomach (Figure 5(b); $P < .01$, <.0001, <.05, resp.). PPAR protein expression did not change with fetal age (Figure 5(c); 26 fetuses, ED59–120).

3.6. Intestine. PPARα and γ mRNA expression did not change with age, but PPARβ decreased ($P < .001$, Figure 6(a), 32 fetuses, ED54–120). PPARα, β, and γ were expressed at similar levels (Table 2). β2M mRNA expression was higher than PPAR (mean Ct ± SEM: β2M = 21.4 ± 0.2, PPARα = 27.5 ± 0.2, PPARβ = 27.4 ± 0.1, and PPARγ = 27.6 ± 0.3). Fetal intestinal PPAR mRNA was not significantly different from either the adult small intestine or the adult colon (Figure 6(b)). PPARα protein expression increased ($P < .001$), while PPARβ and γ proteins did not change with fetal age (Figure 6(c); 29 fetuses, ED57–120).

3.7. Adrenal. PPARγ mRNA decreased with age ($P < .05$), while PPARα and β remained unchanged (although $P = .0503$ for PPARβ; Figure 7(a), 46 fetuses, ED54–120). PPARα and β mRNAs were more highly expressed than PPARγ ($P < .001$, Table 2). β2M mRNA expression was higher than PPAR, (mean Ct ± SEM: β2M = 24.2 ± 0.3, PPARα = 29.5 ± 0.3, PPARβ = 29.2 ± 0.2, and PPARγ = 32.1 ± 0.3). Fetal and adult PPARα and β mRNAs were not different, but PPARγ was lower in fetal adrenal ($P < .05$; Figure 7(b)). PPARα and β protein expression decreased with fetal age ($P < .05$, $P < .001$, resp.; Figure 7(c); 36 fetuses, ED67–120).

3.8. Spleen. PPAR mRNA expression did not change with age (Figure 8(a), 23 fetuses, ED67–125). PPARγ was the most

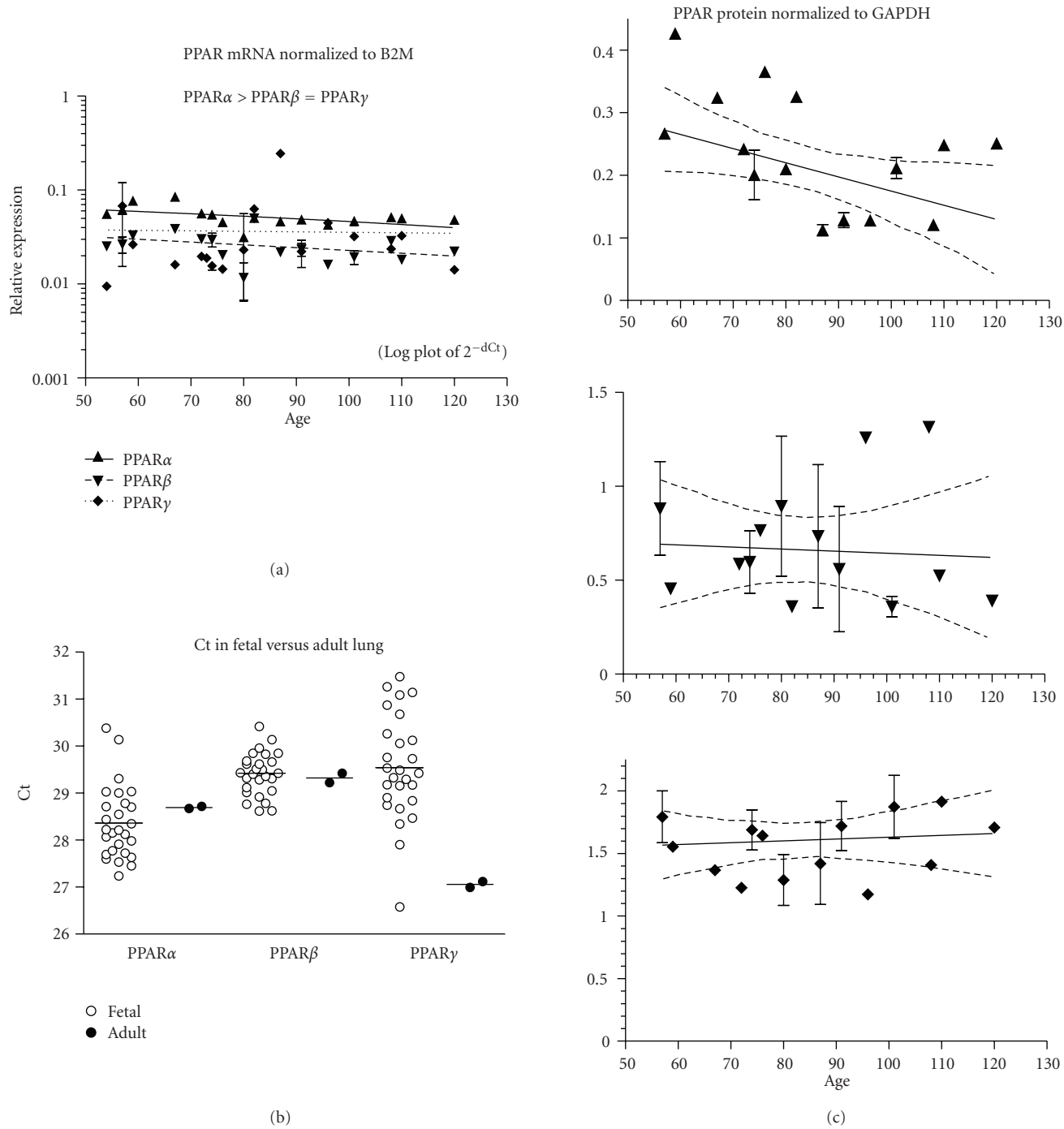


Figure 3: Lung. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult lung. Each symbol represents the mean Ct value of 2 replicates for each fetal (open circles) sample and adult (filled circles) individual replicates are shown (overall mean for each group is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

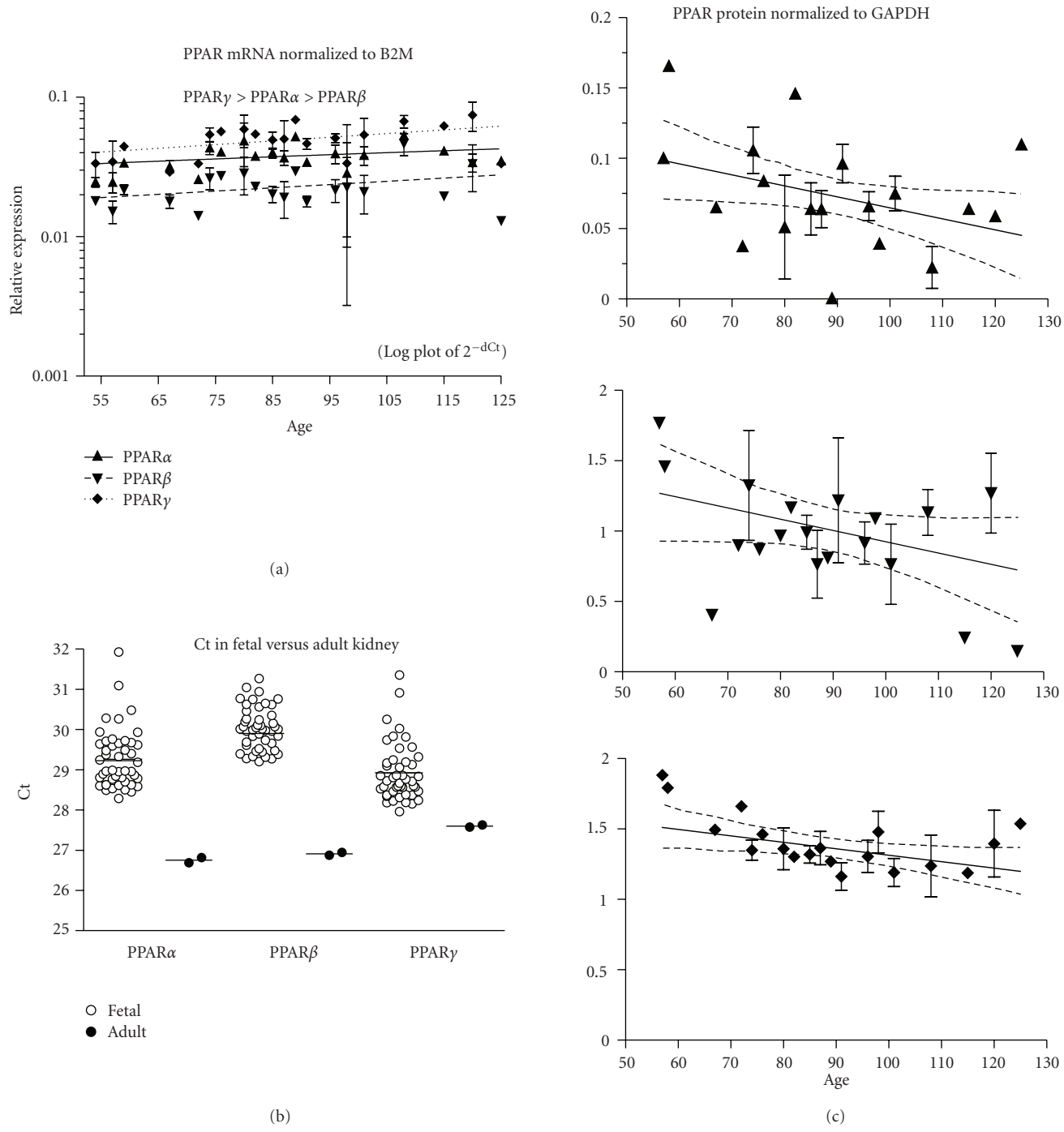


Figure 4: Kidney. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult kidney. Each symbol represents the mean Ct value of 2 replicates for each fetal (open circles) sample and adult (filled circles) individual replicates are shown (overall mean for each group is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

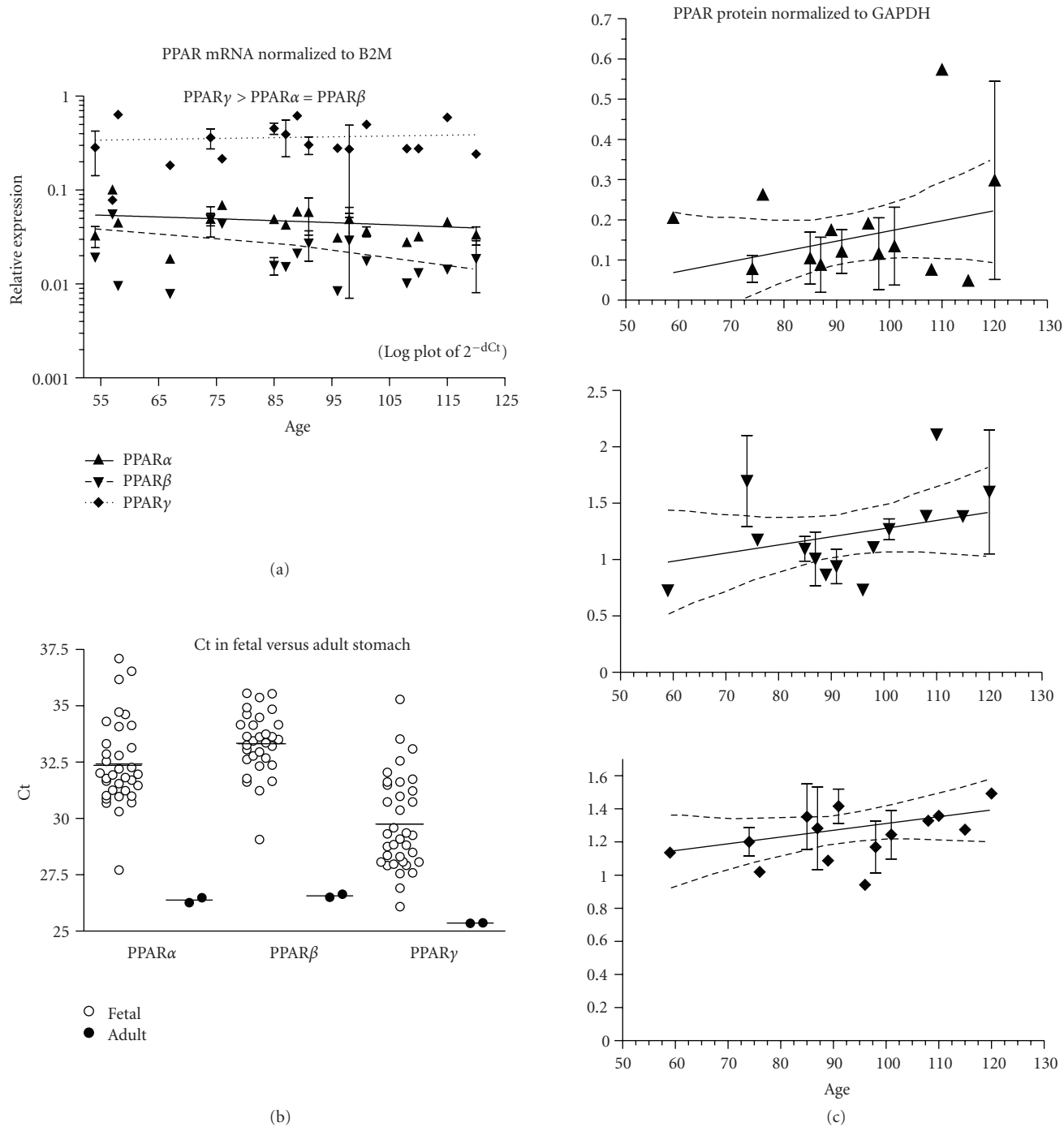


Figure 5: Stomach. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult stomach. Each symbol represents the mean Ct value of 2 replicates for each fetal (open circles) sample and adult (filled circles) individual replicates are shown (overall mean for each group is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

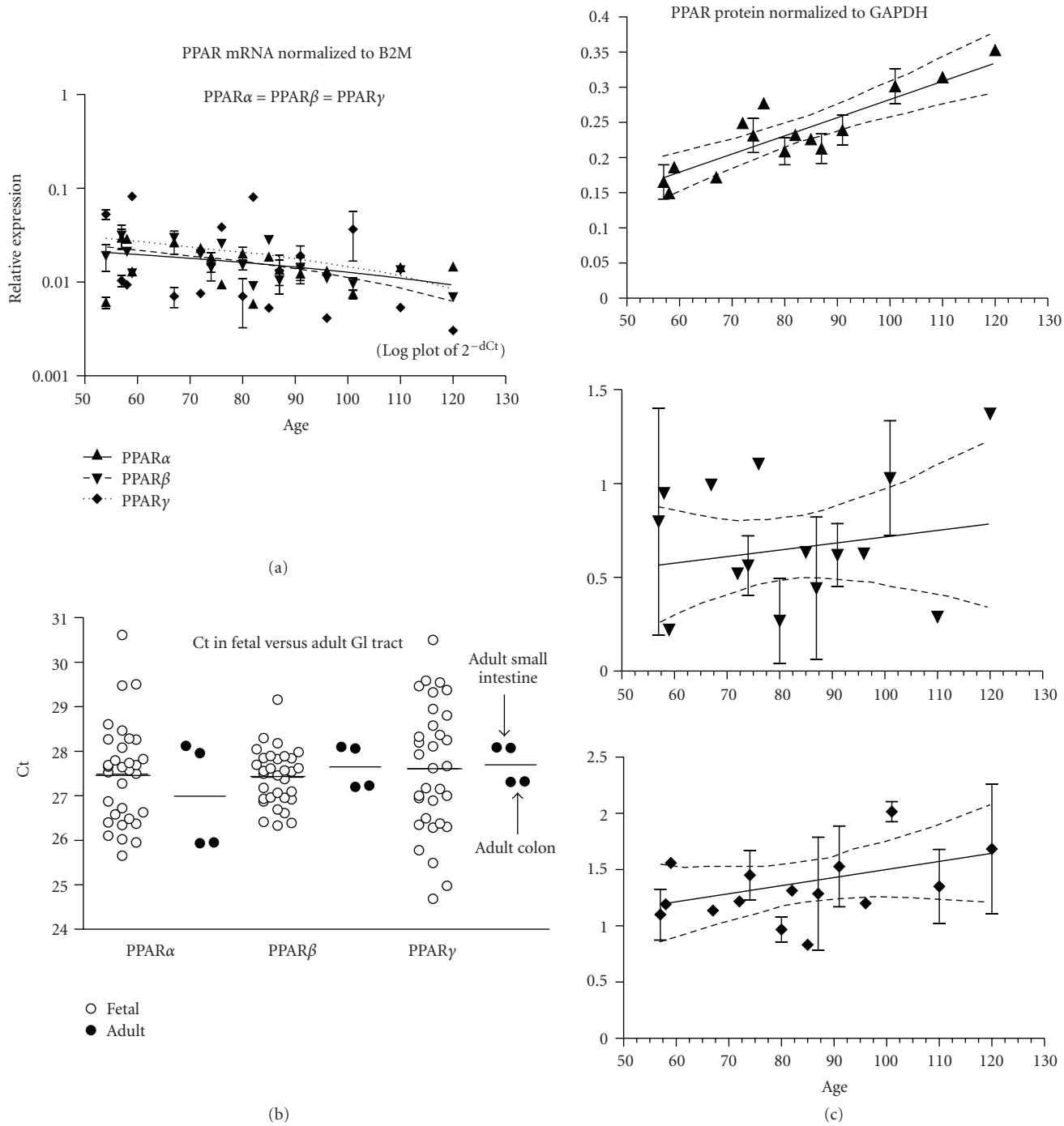


Figure 6: Intestine. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult small intestine and colon (filled circles show the 2 replicates of small intestine above and 2 replicates of colon below the line indicating the mean of the combined tissue values). Each open symbol represents the mean Ct value of 2 replicates for each fetal sample (overall mean is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density is normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

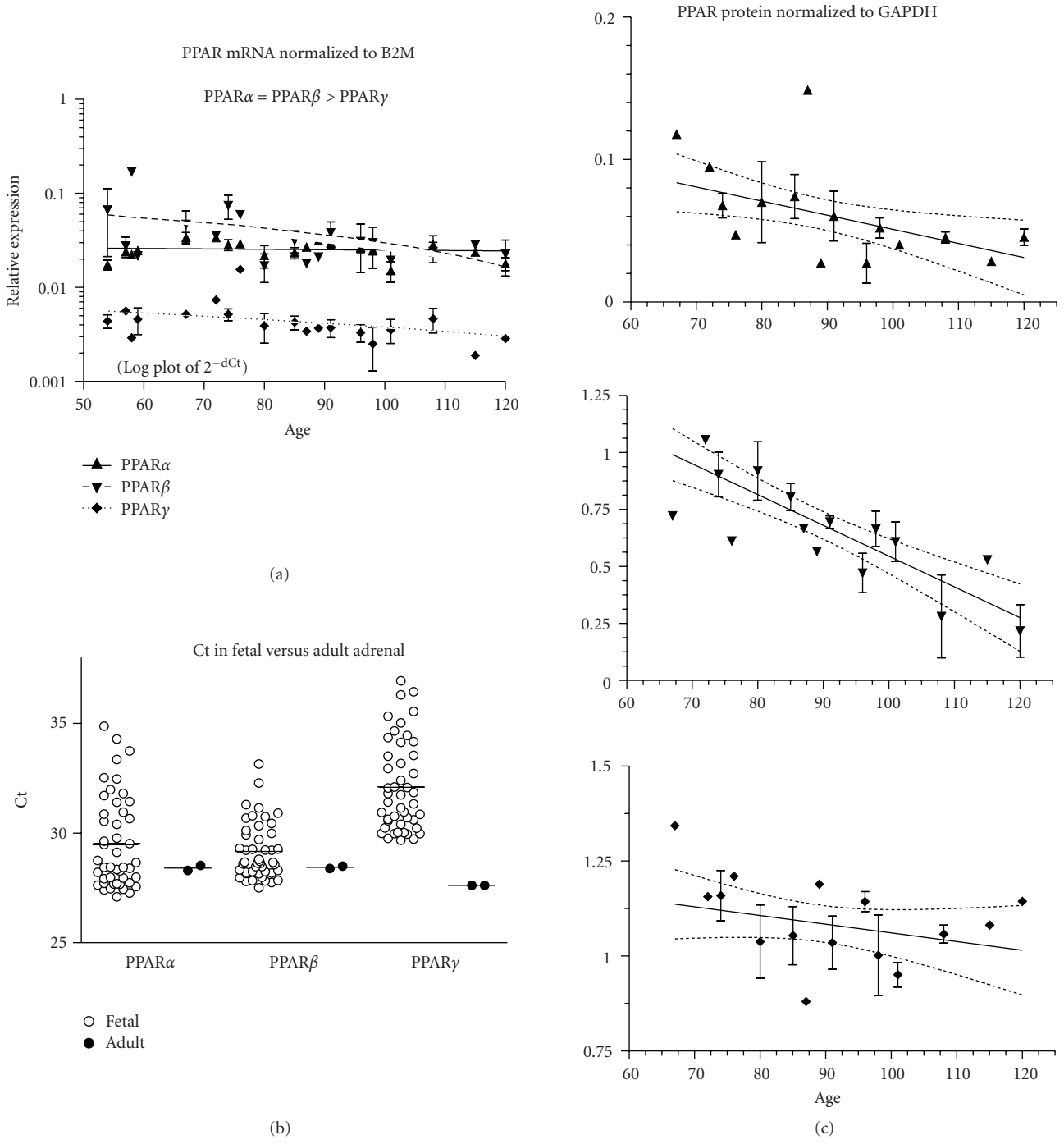


Figure 7: Adrenal. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult adrenal. Each symbol represents the mean Ct value of 2 replicates for each fetal (open circles) sample and adult (filled circles) individual replicates are shown (overall mean for each group is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density is normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

Table 1: Relative RNA[1] expression for each subtype (listed from highest to lowest mean expression), characteristics[2] of RNA and protein expression across age, and fetal mRNA expression relative to adult.

RNA abundance: high to low	RNA change with age	Fetal versus adult mRNA	Protein change with age
	PPARα		
Intestine	NS	NS	Increase
Liver	Increase	NS	NS
Lung	NS	NS	Decrease
Heart	NS	Lower	NS
Kidney	NS	Lower	Decrease
Adrenal	NS	NS	Decrease
Thymus	NS	NS	NS
Stomach	NS	Lower	NS
Spleen	NS	Lower	NS
	PPARβ		
Intestine	Decrease	NS	NS
Adrenal	NS	NS	Decrease
Lung	NS	NS	NS
Kidney	NS	Lower	NS
Thymus	NS	Higher	NS
Liver	Increase	NS	NS
Heart	Decrease	Lower	NS
Spleen	NS	Lower	NS
Stomach	NS	Lower	NS
	PPARγ		
Thymus	NS	NS	NS
Intestine	NS	NS	NS
Spleen	NS	NS	NS
Liver	NS	Higher	NS
Kidney	NS	NS	Decrease
Lung	NS	Lower	NS
Stomach	NS	Lower	NS
Heart	NS	Lower	NS
Adrenal	Decrease	Lower	NS

[1]Relative RNA expression based on mean Ct for all samples across all ages for each tissue, listed from highest to lowest mean expression for each subtype.
[2]Change in RNA and protein expression with age shown as increased, decreased, or not significant (NS) and fetal mRNA expression compared to adult expression shown as higher, lower, or not significantly (NS) different from adult.

highly expressed isotype ($P < .001$, Table 2). β2M mRNA expression was higher than PPAR (mean Ct ± SEM: β2M = 25.6 ± 0.2, and PPARα = 33.5 ± 0.3, PPARβ = 32.9 ± 0.2, PPARγ = 28.3 ± 0.3). Fetal PPARα and β mRNAs were lower than in the adult ($P < .01$, Figure 8(b)). PPARα, β, and γ protein expression did not change with fetal age (Figure 8(c); 11 fetuses, ED85–125).

3.9. Thymus. PPAR mRNA expression did not change with age (Figure 9(a), 11 fetuses, ED74–120). PPARγ mRNA expression was higher than PPARα or β ($P < .001$), and that of PPARβ was higher than PPARα ($P < .01$). β2M mRNA expression was higher than PPAR (mean Ct ± SEM Ct: β2M = 23.1 ± 0.4, PPARα = 31.3 ± 0.3, PPARβ = 30.0 ± 0.1, and PPARγ = 27.5 ± 0.3). PPARβ fetal mRNA expression was higher than in the adult ($P < .05$, Figure 9(b)). PPAR protein expression did not change with fetal age (Figure 9(c); 5 fetuses, ED101–120).

3.10. Comparison of PPARα, β, γ Expression Levels in Different Tissues. Table 1 lists the tissues in an order based on the level of RNA expression in fetal tissues such that the first tissue listed for each subtype has the highest and the last in the list has the lowest expression. The ranking for RNA expression is based on the mean Ct across all ages for each tissue. Expression of PPARα mRNA is the highest in the intestine, liver, and lung and is relatively low in stomach and spleen. PPARβ was the highest in intestine, adrenal, and lung, while expression in spleen and stomach was relatively low. PPARγ was the highest in thymus, intestine, and spleen, but

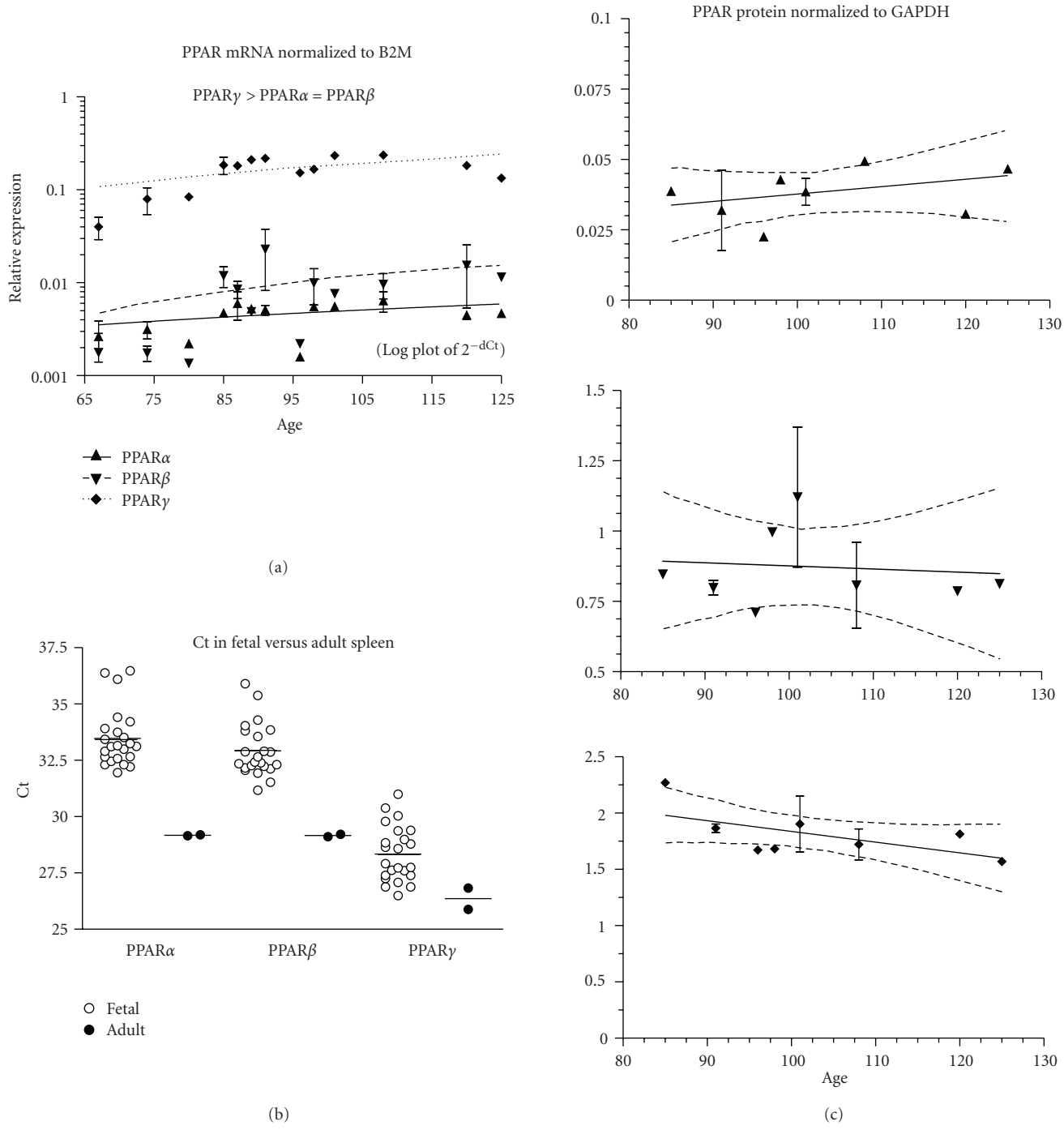


Figure 8: Spleen. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult spleen. Each symbol represents the mean Ct value of 2 replicates for each fetal (open circles) sample and adult (filled circles) individual replicates are shown (overall mean for each group is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density is normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, and down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

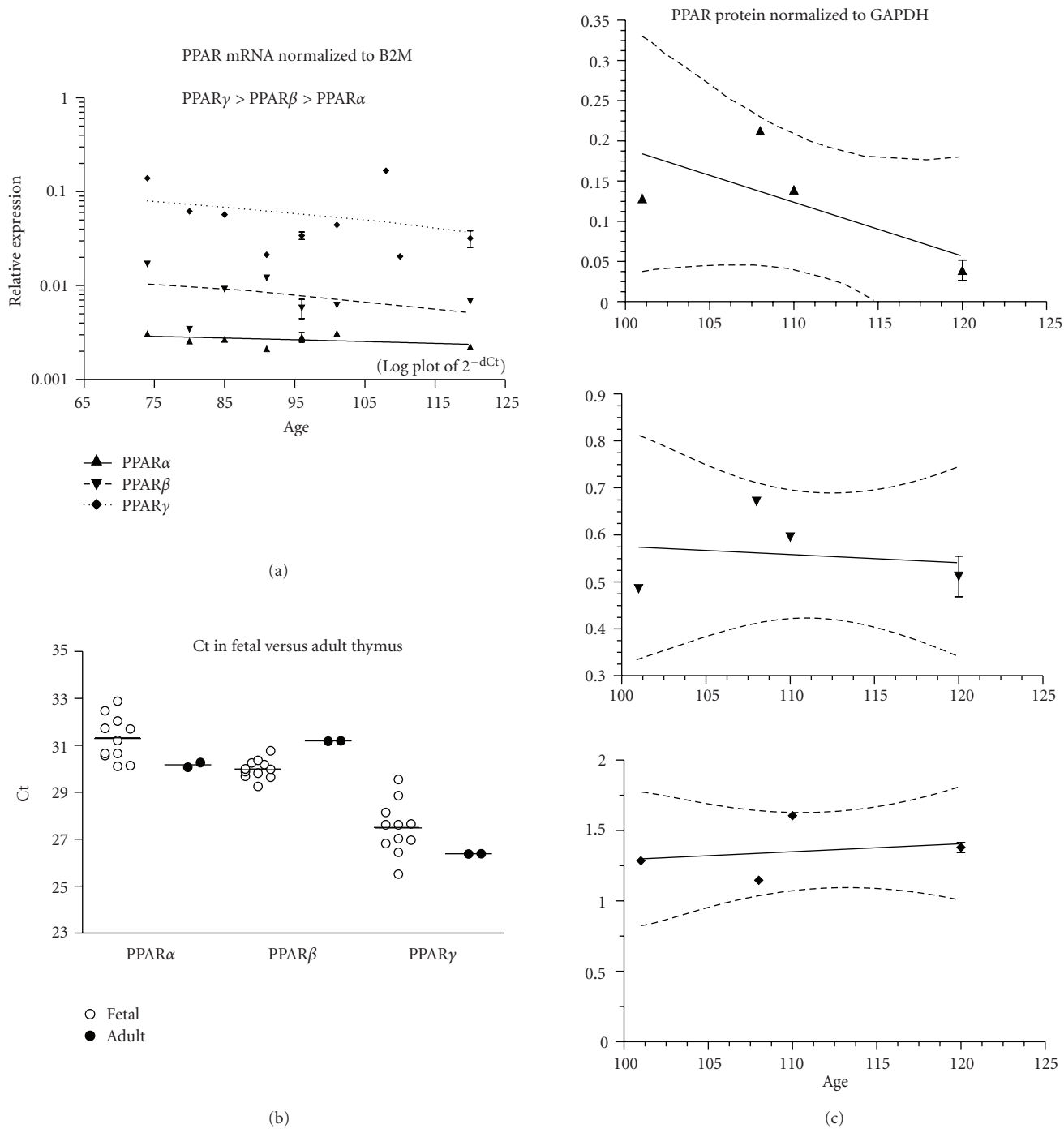


Figure 9: Thymus. (a) The expression of PPARα, β, and γ mRNA is shown across the fetal age range. Log plot of mean ± SEM Ct is normalized to β-2-microglobulin (B2M). (b) The fetal expression of PPARα, β, and γ is shown relative to expression in adult thymus. Each symbol represents the mean Ct value of 2 replicates for each fetal (open circles) sample and adult (filled circles) individual replicates are shown (overall mean for each group is shown as a horizontal line). (c) PPARα, β, and γ protein expression is shown across the fetal age range. Western blot density is normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). Up arrowhead indicates PPARα, and down arrowhead PPARβ, and diamond PPARγ. If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines in graphs of C are the 95% confidence interval.

Table 2: Relative RNA expression of PPAR isotypes within each tissue.

Intestine	$\alpha = \beta = \gamma$
Liver	$\alpha = \gamma > \beta$
Lung	$\alpha > \beta = \gamma$
Heart	$\alpha > \beta > \gamma$
Adrenal	$\alpha = \beta > \gamma$
Thymus	$\gamma > \beta > \alpha$
Spleen	$\gamma > \alpha = \beta$
Kidney	$\gamma > \alpha > \beta$
Stomach	$\gamma > \alpha = \beta$

Relative expression based on mean Ct across ages for all samples of each tissue.

was poorly expressed in heart and adrenal. Among all the tissues, intestine was unique in having high expression of all three subtypes, although lung expressed high levels of both PPARα and β. Stomach poorly expressed all three subtypes relative to the other tissues. Spleen showed weak expression of both PPARα and β, while expression of PPARβ and γ was weak in the heart.

Comparison of the relative levels of protein expression for each PPAR subtype is not presented. The expression of GAPDH did not change with age, making it a suitable loading control for normalization of the PPAR densitometry values from the Western blots for each tissue across age (data not shown); however, GAPDH expression was substantially different between the tissues and that makes it inappropriate to compare or rank the normalized expression between the different tissues. Even considering that the levels of GAPDH were not uniformly expressed in the different tissues, it is clear that there were different levels of PPARα, β, and γ proteins in the various tissues. This can be seen in Figure 10 which illustrates the expression of PPAR proteins in all 9 tissues on single blots for each subtype. In these assays, all 9 tissues were present on each blot and, to the extent possible, the tissues on each blot were from the same 91-day old fetus. Thymus was only available from 101-, 108-, and 110-day old fetuses. Three of the cross-tissue assays (each using tissues from different fetuses) were run for each PPAR subtype and examples of the multiple tissue blots are shown in Figure 10.

4. Discussion

This study provides new information regarding the expression of PPAR subtypes during human fetal development. PPARα, β and γ are expressed in the human fetus from embryonic days 54 to 125. Protein and mRNA for all three PPAR subtypes were detected in the 9 tissues examined in this study. In some organs, the expression of mRNA or protein changed during the developmental period examined. Relative levels of mRNA expression of the PPAR subtypes varied by tissue. In some organs, the level of mRNA expressed was comparable to or higher than that of the adult tissue.

Human fetal expression of PPAR subtypes can be considered similar to the expression patterns reported for the laboratory rodent, reviewed in [26]. In mouse and rat, PPAR mRNA and/or protein was detected during prenatal and postnatal development for liver, kidney, heart, lung, adrenal, spleen, vertebra, tissues of the central nervous system (CNS), brain, adipose, fat, muscle, and skin. The patterns of expression varied by tissue and were dependent on developmental stage. It is difficult to make specific comparisons between developmental patterns of PPAR expression in the laboratory animal and the human fetal tissues of this study as comparisons between comparable developmental stages become complicated following the end of organogenesis [26]. In the present study, the period of human fetal development ranged from about 8 to 18 weeks, a period following organogenesis and encompassing the fetal stage of rapid growth, differentiation, and functional maturation of the organ systems. The end of organogenesis and beginning of the fetal period are generally considered to occur at the end of the eighth week of gestation [37] and a landmark of the entry to the fetal stage is the fusion of the secondary palate. Palatal fusion in human fetuses begins around embryonic day 54 and is generally complete in the 56-57-day-old fetus [37, 38]. In the mouse and rat, palatal fusion occurs on ED14-15 and 16-17, respectively, although this can vary by a day or two depending on the strain. Thus, it may be reasonable to consider the ED14 mouse, ED16 rat, and the ED54–56 human fetal tissues to be at comparable developmental stages for purposes of comparison of PPAR expression. Restricting the discussion to that specific developmental period (end of organogenesis marked by palatal fusion), the comparisons of human and rodent PPAR expression are somewhat limited. Overall, as discussed below, there are similarities, and also some differences, in the expression of PPAR in rodent and human fetuses at the end of organogenesis.

In the ED15.5 rat liver, moderate levels of mRNA for PPARα and β were found and PPARβ protein was reported in ED15 mouse liver and PPARγ2 protein was detected at a slightly earlier stage (ED13) in mouse liver [39, 40]. In the present study, PPARα was highly expressed in the human fetal liver and relatively abundant compared to other tissues (only intestine was higher). When evaluated across all ages, PPARα and γ were more abundant than β in liver.

Rat heart and lung expressed PPARα and β, and PPARβ protein expression is reported for mouse heart and lung [40, 41]. In the present study, human fetal lung and heart had high expression of PPARα and lung strongly expressed PPARα and β relative to the other organs. In human fetal heart and lung, PPARα was more abundant than β or γ, and in heart γ was the subtype with the least expression.

The ED15.5 rat and 14.5 mouse kidneys expressed PPARα mRNA. PPARβ mRNA was found in rat and PPARβ and γ were weakly detected in the mouse kidney [41, 42]. In human fetal kidney, PPARγ was expressed at higher levels than α or β, and β was the least abundant subtype in kidney.

We are not aware of any published data regarding expression of PPAR in thymus or spleen of the developing rodent. In the human fetal spleen, PPARα and β were expressed at low but equivalent levels and PPARγ was the most abundant subtype with relatively high expression (only those of thymus and intestine were higher). In human fetal thymus, PPARγ mRNA was very abundant (higher than in

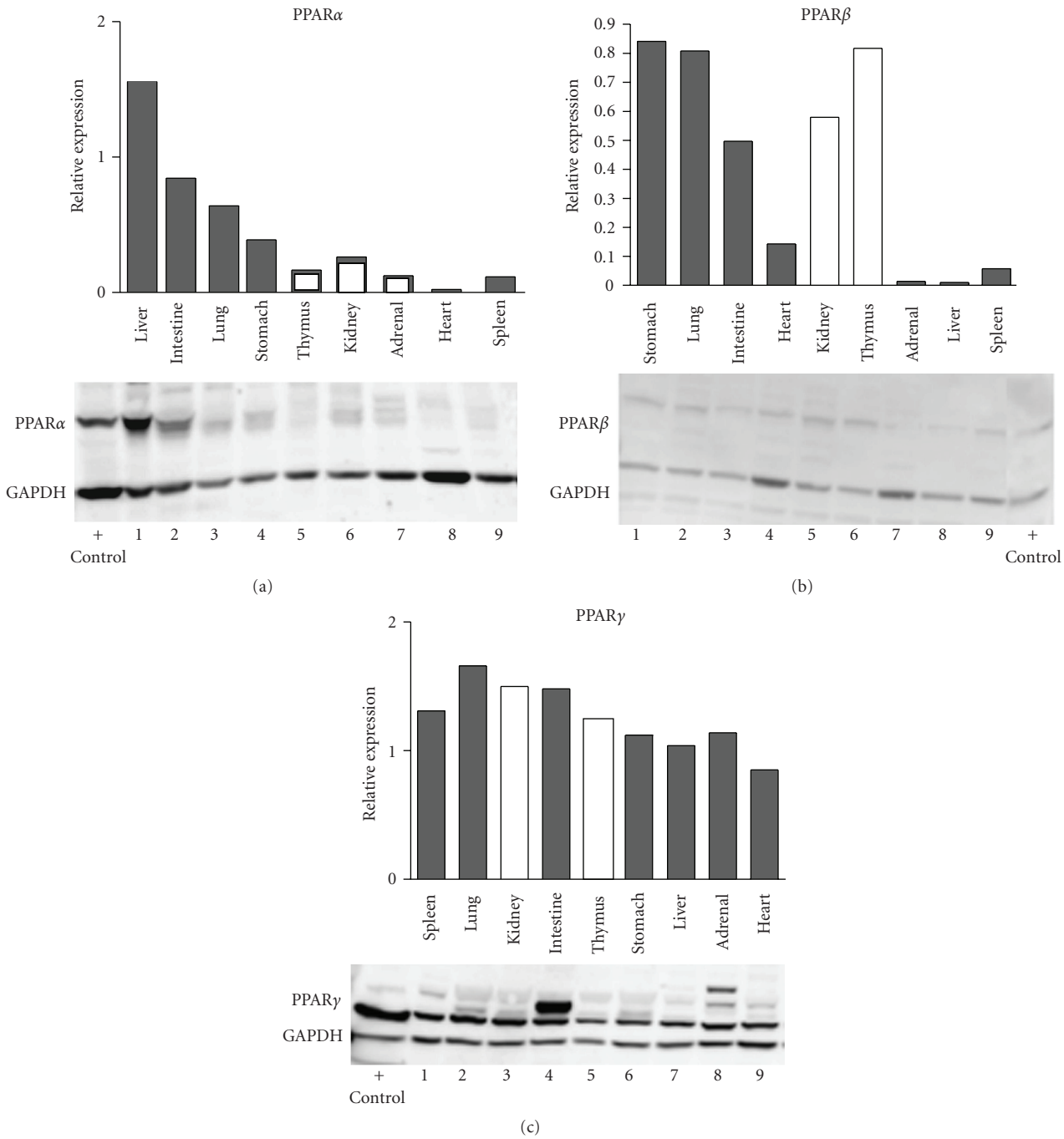


Figure 10: Western blots are shown in which all 9 tissues are present on each blot. On the PPARα blot, all tissues shown by dark bars were from a single 91-day-old fetus, and adrenal and kidney (white bars) were from different 91-day-old fetuses, and thymus was from a 101-day-old fetus. The blots for PPARβ and PPARγ used samples from a 91-day-old fetus (dark bars, the same set of samples for both PPARβ and γ) with kidney and thymus samples (white bars) from different fetuses (91 and 108 days, resp.). Blot images are labeled to show the location of the PPAR band, the GAPDH band, and lane containing the positive control (Hep G2 whole cell extract, Jurkat cell nuclear extract, and U937 whole cell extract, for expression of PPARα, β, or γ, resp.). The densitometry data (PPAR expression normalized to GAPDH) for each gel is shown above the blot image. Lanes 1–9 contain the samples listed on the x-axis of the bar graphs.

any other tissue) and PPARβ and α were detected at lower levels than PPARγ.

In rat GI tract, mRNAs for PPARα and β, but not γ (reported as not detected), were expressed, and PPARβ protein was reported in mouse GI tissue [40, 41]. The present study found high expression in intestine for all PPAR subtypes relative to the other organs, and PPARα, β, and γ mRNAs were at equivalent levels. Stomach, which was examined separately, had lower expression of all subtypes relative to intestine, and PPARγ was the most highly expressed

subtype in stomach. Huin et al. [30] examined PPAR protein expression in the fetal human digestive tract (aged 7 to 22 weeks) using immunohistochemistry and found spatial and temporal patterns of expression in esophagus, stomach, jejunum, ilium, and colon. In the present study, using qPCR and Western blotting methods, no change with age was detected in stomach for expression of PPARα, β, or γ. Huin's report found slightly less PPARα at 19 weeks compared to 12 and 15 weeks of age, while PPARβ and γ were slightly higher at 15 and 16 weeks, respectively, than at 12 or 19 weeks of gestation. The 19-week observations of Huin's study are just outside the age range of the present study, but the slight changes in protein reported by Huin differ from our observations of mRNA and protein at the earlier ages. The intestinal expression of PPAR reported for the various regions observed in Huin's study is similar as an overall pattern to that found in the present study; however, in the present study it was not possible to separate regions of the intestinal tract. Huin reported increasing PPARα in the ileum from 12 to 22 weeks of age, similar to the increase with age in PPARα protein observed from 8 to 18 weeks in the present study. Similarly, Huin reported that PPARβ and γ protein expression in the jejunum and ilium was similar across time (7–16 and 12–22 weeks, resp.), and the present study also found no significant change in protein expression of PPARβ or γ with age.

An important finding of the present study is that fetal tissues can express PPAR at levels equivalent to those of the adult tissues (or higher in the case of PPARγ in liver and PPARβ in thymus). However, some caution is needed as the adult data for each tissue (with the exception of liver) is based on a pooled total RNA sample from 3 donors and it is not known whether a similar outcome would be derived from a larger number of adult donors. However, in the case of liver, the data from 23 individuals supported the data from the pooled sample, that is, expressions of PPARα and β were not significantly different in adult and fetal livers. However, the pooled sample did not detect the increased expression of PPARγ in fetal liver relative to the adult, as observed in the 23 individual liver samples. Thus, the adult versus fetal comparison provides data that were previously not available and represent the only information for this endpoint. However, it is important to recognize that comparisons of these data with additional analyses from larger adult tissue sets would be desirable.

In summary, this study is unique in providing substantial information on the expression of PPARα, β, and γ during human fetal development. Among the strengths of the study are the acquisition of both protein and mRNA data from the same samples, the inclusion of multiple tissues from most fetuses, and the large number of individuals represented in the sample set. Representation of tissues across a range of ages supported an evaluation of whether PPAR expression changed as development progressed. The qPCR approach supported estimation of the relative expression of subtypes within a tissue as well as supporting comparisons of expression of each subtype across the different tissues. As mentioned in the introduction, an important aspect of this study was to provide information for use in assessing the potential for the human fetus to respond to PPAR agonists. Studies in human fetal tissues of responses to PPAR agonists are generally not feasible; thus, it is important to at least have information on the developmental expression of PPAR and how that compares to adult expression. This study contributes to our knowledge regarding the expression of PPAR during development and compares fetal and adult PPAR expression. An important finding of the study is that fetal tissues can have expression levels equivalent to those of the adult tissues (or higher in the case of PPARγ in liver and PPARβ in thymus). The role of PPAR subtypes in the developing fetus remains unclear, but it is likely that these nuclear receptors have roles similar to those described for adult tissues, including regulation of energy homeostasis as well as lipid and glucose utilization. During the fetal stages examined in this study, the organs undergo rapid growth, differentiation, and acquisition of functionality. Exogenous agents that alter PPAR signaling in the adult, such as environmental agents, chemicals, or drugs, are capable of affecting lipid and glucose utilization, cholesterol biosynthesis, and other metabolic pathways, and these attributes make PPAR signaling an attractive target for pharmaceuticals directed at management of disease states (diabetes, metabolic syndrome, hyperlipidemia) [4, 40]. This study showed that PPAR subtypes are expressed during human fetal development in many organs and it is likely that PPAR expression and function during development are tightly regulated. It is not clear whether specific agents perturb PPAR expression or function in the fetus, whether such perturbations will have consequences or whether effects might emerge at or persist through much later life stages. However, the demonstration of expression of PPARα, β, and γ in nine major organs during human fetal development renders consideration of such issues highly relevant.

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Drs. Michael Devito and Chris Corton (US EPA) generously made available samples of adult human liver, which they obtained from CellzDirect, Inc. (Durham, NC). The authors also appreciate the assistance of Cynthia J. Wolf and Jason Stanko with the animal work in this study.

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Erratum

Erratum to "Peroxisome Proliferator-Activated Receptors Alpha, Beta, and Gamma mRNA and Protein Expression in Human Fetal Tissues"

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In the above-mentioned paper, the expression of PPARγ was incorrectly determined based on the quantification of a 50 kD band on the Western blots. This band aligned with what was believed to be a positive control band in the U937 whole cell extract, a control reagent recommended by the supplier of the primary antibody, SC-7196 (Santa Cruz Biotechnologies, Santa Cruz, CA). Based on recent information described by Foreman et al. [1], it is clear that this band is not PPARγ but was a nonspecific immunoreactive protein detected by SC-7196. This nonspecific protein was abundantly detected by SC-7196 in U937 and COS-1 cells as well as across all human fetal protein samples. Immunoprecipitation of COS-1 cell lysate using agarose-conjugated SC-7196 resulted in a single band on a Coomassie Blue-stained gel. This band was subjected to digestion, peptide extraction, and sequence analysis using MALDI-MSMS, and the protein was identified as cytoplasmic actin with a decisive score (human SwissProt database, 60% protein coverage using the 15 highest scoring peptide groups and two lower scoring but acceptable peptides).

A specific band for PPARγ, (calculated molecular weight for human PPARγ1 = 54.55 kD) was identified on our Western blots by performing new experiments in which in vitro translated human PPARγ1 (provided by J. Peters, Pennsylvania State University) was compared with human fetal tissue lysates (Figure 1). These experiments also included COS-1 cell lysate as a negative control and U937 cell lysate. The Western blots of the fetal tissues were all reanalyzed using the ~55 kD band that aligned with the in vitro translated human PPARγ1. Based on this reanalysis, the expression of PPARγ protein shown in (c) of Figures 1–9 are replaced by Figure 2. The data summary described in Table 1 of the above-mentioned paper regarding the change in PPARγ protein expression with fetal age is replaced by Table 1.

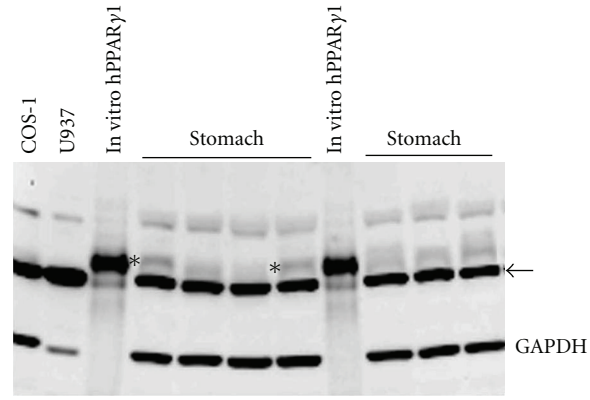

Figure 1: Western blot showing the comparison of banding patterns in COS-1 cell lysate, U937 cell lysate, in vitro translated human PPARγ1, and tissue lysate from human fetal stomach samples. The ~55 kD band of human PPARγ1 and corresponding band in stomach tissue lysate is marked with an asterisk (*). The nonspecific, cytoplasmic actin band is marked with an arrow.

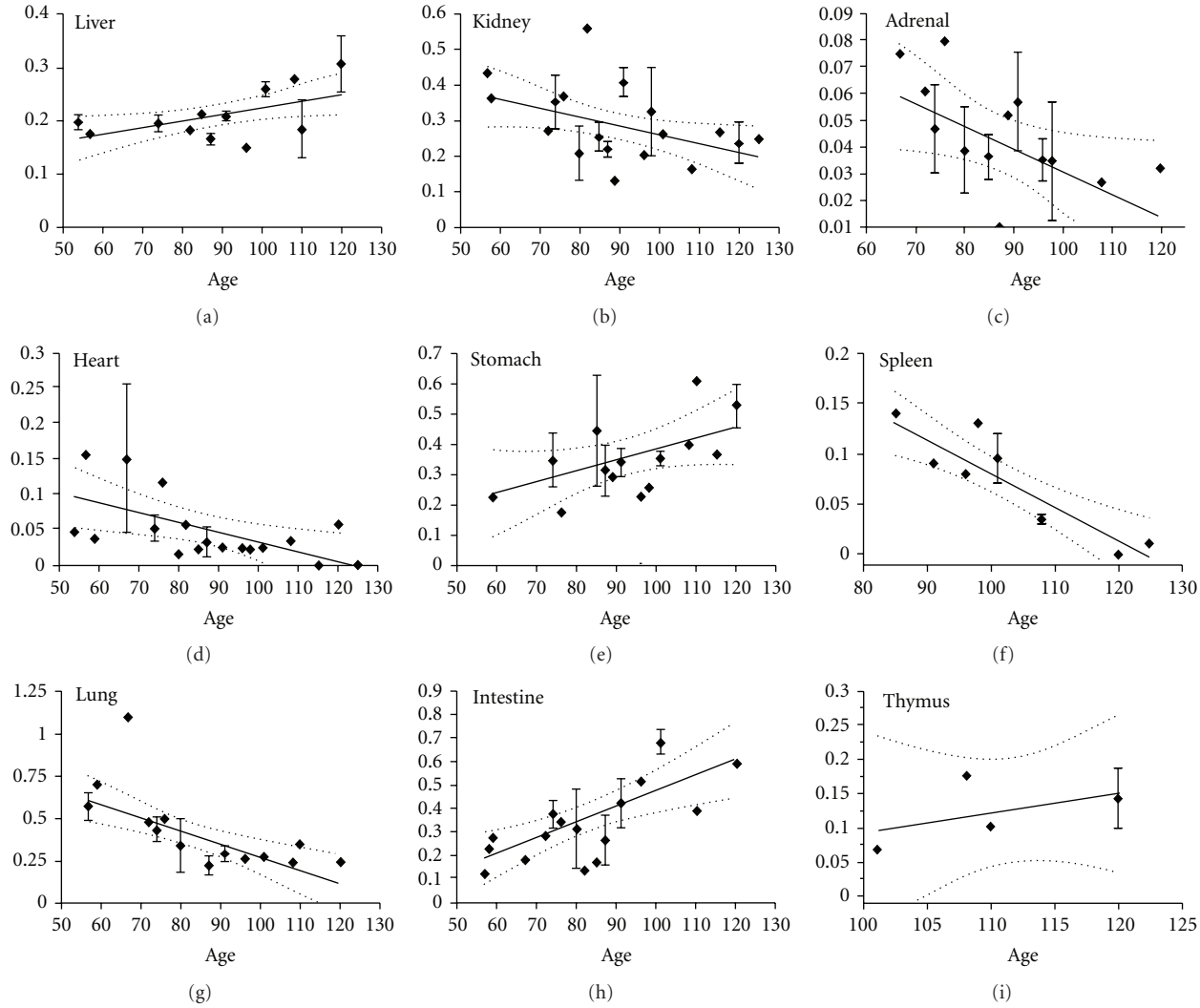

Figure 2: PPARγ protein expression is shown across the fetal age range for each tissue. Western blot density normalized to glyceraldehyde-3-phophate dehydrogenase (GAPDH). If only one sample was available for a particular age, then an error term could not be calculated and no SEM bar is shown. Regression analysis evaluated change with age. Dashed lines are the 95% confidence interval.

Table 1

Tissue	Protein change with age	
Thymus	NS	
Intestine	Increase	$P < .01$
Spleen	Decrease	$P < .001$
Liver	Increase	$P < .05$
Kidney	Decrease	$P < .05$
Lung	Decrease	$P < .001$
Stomach	NS	
Heart	Decrease	$P < .05$
Adrenal	Decrease	$P < .05$

NS = no significant change with age.

The authors regret this unexpected error. The clarification of the recognition patterns of this primary antibody should be of value to investigators interested in detecting PPARγ protein.

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Review Article

The Role of PPARα Activation in Liver and Muscle

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PPARα is one of three members of the soluble nuclear receptor family called peroxisome proliferator-activated receptor (PPAR). It is a sensor for changes in levels of fatty acids and their derivatives that responds to ligand binding with PPAR target gene transcription, inasmuch as it can influence physiological homeostasis, including lipid and carbohydrate metabolism in various tissues. In this paper we summarize the involvement of PPARα in the metabolically active tissues liver and skeletal muscle and provide an overview of the risks and benefits of ligand activation of PPARα, with particular consideration to interspecies differences.

1. Introduction

Dietary fatty acids (FAs) are not only important for membrane structures and in signalling processes, but also have the ability to influence gene expression by binding to specific transcription factors [1]. One receptor family that acts as mediators to influence transcription according to nutritional state is the peroxisome proliferator-activated receptor (PPAR) family. There are three isoforms of PPAR receptors that have specific, but also overlapping target genes: α, β/δ, and γ [2–4]. Early on PPAR activity was thought to mainly influence lipid metabolism, inflammation, and glucose homeostasis. Later it became clear that PPARs also play a role in modulating the processes of cell proliferation and differentiation, apoptosis, and aging [5–8]. The receptors show a nuclear localization in the form of a heterodimer with the retinoid X receptor (RXR). A ligand activated PPARα-RXR heterodimer regulates the transcription of genes by binding to their peroxisome proliferator response elements (PPREs), a process called "transactivation" [9–11]. Besides, a mechanism based on "transrepression" has been described and is reviewed in [12]. The anti-inflammatory actions of PPARα ligands are mostly thought to be based on "transrepression" by the negative interference of PPARα with other transcription factor pathways [13, 14].

Here we focus on the first identified PPAR receptor, PPARα [15], and its activation in different tissues and physiological states in humans and mice. It is expressed at elevated levels in tissues with high metabolic rates, such as the liver, heart, skeletal muscle, kidney, and also in the intestine [12, 16]. Additionally, it is present in cells of the immune system (e.g., macrophages, monocytes, and lymphocytes) [17–19]. The receptor has a central role in fatty acid oxidation, lipid and lipoprotein metabolism, inflammatory responses, and oxidative stress. Its position in the centre of energy balance, lipid metabolism, and inflammation makes it an important factor in the development of obesity-related diseases, and therefore, presents a possible target to influence metabolic disorders. Ligands include saturated and unsaturated FA and their derivatives, hypolipidemic fibrates (ciprofibrate, clofibrate, fenofibrate, and gemfibrozil), and modified fatty acids (e.g., tetradecylthioacetic acid, TTA), as well as xenobiotics [20–22]. In particular during fasting, when free FAs are released into the blood, endogenous lipid-activation is of importance. The importance of PPARα in the cellular metabolic response to fasting was clearly shown in PPARα-null mice [23]. Whereas under normal conditions, these mice do not display a strong phenotype, the absence of PPARα causes lipid accumulation in liver and heart, hypoglycemia, hypothermia, ketonuria, and elevated free fatty acids during fasting ultimately leading to premature death [23]. In contrast, wildtype mice adapt to fasting by induction of hepatic and cardiac PPARα target genes that results in increased FA uptake and oxidation [24].

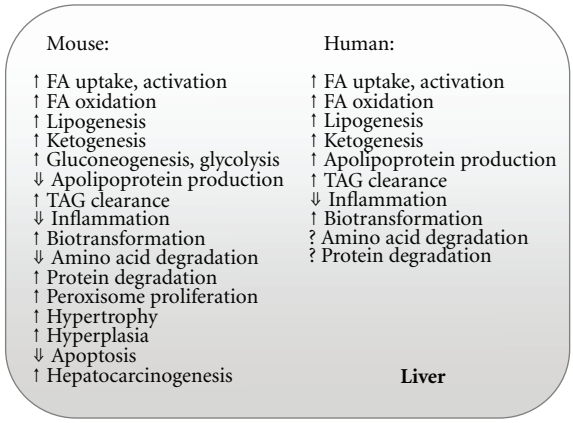

Figure 1: Examples of the multiple metabolic effects of PPARα activation in mouse or human liver. FA, fatty acid; TAG, triacylglycerol.

A great number of animal studies have demonstrated beneficial effects of specific PPAR activation in counteracting metabolic disorders. An increasing number of human studies supports the findings obtained in animal studies. When it comes to PPARα activation, however, it has become clear that not all results obtained in mice can be extrapolated to humans and caution is warranted in predicting tissue-specific effects.

This paper will focus on the tissues liver and skeletal muscle exploring tissue-specific effects of PPARα activation and stress the differences of human- and mouse-based studies.

2. PPARα in Liver

There are substantial differences between human and mouse target gene expression in terms of the effect of PPARα activation in the liver (Figure 1). Overall, the effect of activation by the PPARα agonist WY14643 is more prominent in mice than in humans [25]. In primary hepatocytes from mice and humans treated with WY14643, only a few target genes were affected similarly in the two species. However, both species share multiple changed gene ontology classes, including lipid metabolism. Individual PPARα regulation was observed for enzymes involved in biotransformation (chemical alterations of compounds in the body), as well as apolipoprotein and bile acid synthesis in human hepatocytes, and glucose homeostasis in mouse hepatocytes [25]. It was proposed earlier that the response might be dampened by quantitative differences of *PPARα* expression or different splice forms of *PPARα*. Indeed, there exist two splice variants of *PPARα* giving rise to an active and inactive receptor in humans [26]. To compare *PPARα* expression levels between human and mouse liver is, however, difficult due to daily variations [27] and differing reports have been published. Some reports show lower *PPARα* expression levels in human than in rodent liver [28–30], while another shows comparable expression levels between the two species [25].

One of the main pathways involving PPARα regulation in mice and humans includes FA metabolism. In mice, PPARα activation is important for FA metabolism through the induction of genes coding for the fatty acid transporter CD36 [31] and the FA binding protein 1 (FABP1) that brings the FAs from the plasma membrane to the nucleus [32]. Another PPARα target gene is carnitine palmitoyl transferase 1 (*Cpt1*), that codes for a protein important for FA transport into mitochondria [25].Whereas CPT1 is localized to the outer membrane, CPT2, that is also regulated by PPARα, is found in the inner mitochondrial membrane. It converts acyl-carnitine to acyl-CoA and is strongly upregulated by PPARα agonists [33]. Most of the genes of FA metabolism are regulated by PPARα in both humans and mice, however *Cd36* is an example of species-specific induction in mice [25].

Genes encoding for mitochondrial proteins of the β-oxidation pathway are induced by PPARα activation, such as acyl-CoA synthetase (*Acs*) coding for an enzyme responsible for activation of FA to their fatty acyl-CoA derivatives. Also genes of the short-, medium-, long- and very-long-chain acyl-CoA dehydrogenases (*Acad -s, -m, -l, -vl*) coding for proteins that catalyze the first step in FA oxidation in a chain length-specific manner, are under the control of PPARα. In addition, the expression of the gene encoding the enzyme acetyl-CoA acyltransferase 2 (ACAA2) involved in the final step of β-oxidation, is PPARα dependent. Furthermore, hepatic carnitine synthesis is enhanced by PPARα activation in mice [34, 35]. Carnitine is a conditionally essential nutrient that plays an important role in mitochondrial long-chain FA import for β-oxidation [36]. In PPARα-null mice, free carnitine levels were drastically suppressed in plasma and several tissues including liver, the primary site of carnitine biosynthesis. This was consistent with reduced hepatic expression of the genes involved in carnitine biosynthesis (*Bbox1*) and transport (*Octn2*) [37]. In an earlier study, Van Vlies and colleagues established a fasting-induced elevation of these genes that is PPARα-dependent [38]. Both studies point to an essential position for PPARα in carnitine metabolism in mice [37, 38]. No similar indications of PPARα-induced carnitine synthesis have been described in humans. However, pigs that also are a nonproliferative species and are considered similar to humans due to their metabolic features, show an increased carnitine production upon fasting [39]. It is therefore likely that also humans will prove to have a similar response.

Peroxisomal fatty acid oxidation is important for the partial oxidation of long, very long, and branched FAs. The first characterized PPARα target gene, acyl-CoA oxidase 1 (*Acox1*) encodes the rate-limiting enzyme of this process [40]. After ACOX1 has introduced a double bond to generate enoyl-CoA and H_2O_2, the bifunctional protein/enoyl-CoA hydratase (BIEN), that carries two enzymatic activities, performs the second step of β-oxidation resulting in 3-ketoacyl-CoA. 3-ketoacyl-CoA is then cleaved by acetyl-CoA acyltransferase 1 (ACAA1) to produce acetyl-CoA [41]. All the above-mentioned genes are under the regulation of PPARα in mice.

In addition to mitochondrial and peroxisomal β-oxidation, ω-hydroxylation occurs in smooth endoplasmic reticulum. In both mice and humans, this process is upregulated by the effect of PPARα on expression of cytochrome P450 4A11 (CYP4A11) [25, 42–44]. The hepatic cytochrome

P450 4A11 catalyzes ω-hydroxylation of medium and long-chain FAs. Subsequently cytosolic dehydrogenases convert them to dicarboxylic acids, which can be further processed by peroxisomal β-oxidation. Human PPARα also is a transcriptional regulator of FA oxidation in the different organelles, but shows overlap with mice rather on the pathway than on the gene level [25]. To conclude, PPARα regulates enzymes important for uptake, traffic to final destination, activation, and oxidation of FAs in the three organelles mitochondria, peroxisomes, and microsomes in both mice and humans.

Paradoxically, at the same time as PPARα activation leads to an increase in FA oxidation, it also augments FA synthesis by affecting gene expression levels of several enzymes involved in lipogenesis. In mice, PPARα stimulates the conversion of malate into pyruvate to generate NADPH for lipogenesis by upregulating the expression of malic enzyme (ME1) [45]. Besides, the Δ5, Δ6, and Δ9 desaturases, rate-limiting enzymes in the synthesis of polyunsaturated FAs (PUFAs) from saturated FAs, are found in increased amounts after PPARα activation [46–48]. The induction of desaturases could help to ensure that there are always enough PUFAs for their diverse functions, including being effective PPARα agonists as proposed by others [46]. Likewise, PPARα activation in human hepatocytes induces the expression of several target genes involved in FA synthesis [25].

Other crucial processes requiring PPARα activation are lipoprotein synthesis and assembly. The impact of PPARα agonist on lipoprotein gene expression in humans or mice is distinct. The use of fibrates in humans leads to reduced plasma triacylglycerol (TAG) levels and increased high-density lipoprotein (HDL) cholesterol levels. In mice, plasma TAG as well as HDL levels are lowered. The liver, besides the intestine, determines the amount of HDL in plasma by regulating HDL synthesis and catabolism. The reason for the species-specific opposite effect of PPARα activation on HDL levels is probably increased production levels of apolipoprotein A-I (APOA1) and APOA2 in humans [49, 50] and suppressed (APOA1) or unchanged (APOA2) expression in mice [51]. These apolipoproteins are part of HDL cholesterol and are crucial for reverse cholesterol transport from peripheral cells to the liver, where excess cholesterol can be eliminated into the bile [52]. The liver is also the place where very low-density lipoprotein (VLDL) particles are assembled and then secreted into the plasma. The VLDL amount in peripheral cells is influenced by lipoprotein lipase (LPL). The hepatic expression of this hydrolase, which mediates VLDL triglyceride lipolysis, is upregulated by PPARα [53]. Moreover, its activity is stimulated by APOA5 and inhibited by APOC3. Activation of PPARα increases *APOA5* [54–56] and decreases *APOC3* [57] transcription, resulting in a plasma TAG lowering effect, thereby, together with increased HDL concentrations, reducing the risk for atherosclerosis in humans [58].

The removal of excess cholesterol from the body is via the bile, a fluid produced in the liver, stored in the gall bladder, and secreted into the small intestine. Cholesterol is eliminated either intact or as bile acids that are steroid acids made from cholesterol. In humans, the two main bile acids synthesized in the liver, are chenodeoxycholic acid (CDCA) and cholic acid (CA) [59, 60]. Due to their amphipathic character they aid in the small intestine for the digestion and absorption of dietary lipids. There is controversy in the literature regarding the regulation of the rate-limiting enzyme in hepatic bile acid synthesis, called cholesterol 7α-hydroxylase (CYP7A1). Some reports suggest a transcriptional upregulation of *Cyp7a1* upon PPARα activation in mice [61, 62]. In particular, the upregulation of *Cyp7a1* under fasting conditions and the downregulation of this enzyme in PPARα-null mice corroborate a PPARα regulatory involvement and suggest increased expression upon fasting-induced PPARα activation [62]. Other studies support a downregulation of this endoplasmic reticulum enzyme upon induction with PPARα agonists in both humans and rodents [63–67]. This could be a potential risk for gallstone formation, if in humans receiving treatment with fibrates, bile acid synthesis is decreased over a longer period of time by a hepatic decrease of CYP7A1 activity. On the other hand, gene expression of sterol 12α-hydroxylase (*Cyp8b1*), an enzyme involved in CA synthesis, is increased under fasting and also with ligand-induced PPARα-activation in both rodents and humans [62, 67, 68]. This protein of the cytochrome P450 family controls the balance between CA and CDCA levels. Upon *Cyp8b1* induction, higher CA concentrations positively influence the bile acid composition by increasing cholesterol solubility.

Important under conditions of extended fasting is the process called ketogenesis. In mice and humans, the production of ketone bodies is under the control of PPARα that upregulates the gene expression of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (*Hmgcs2*), coding for the rate-limiting enzyme of ketogenesis [25, 69, 70]. Of particular importance in regulating ketogenesis, in addition to FA oxidation, TAG clearance, and de novo lipogenesis is the 'hormone-like' fibroblast growth factor 21 (FGF21) [71–73]. Its hepatic expression is PPARα-dependent and is induced by fasting, a ketogenic diet, and WY14643 [25, 71, 74, 75]. FGF21 positively influences lipid and glucose metabolism, in addition to insulin sensitivity in animals [76].

Hepatic gluconeogenesis is also regulated during fasting, when the liver changes from glucose uptake and glycogen synthesis to glucose production. The chain of reactions converting glycerol, lactate, or glucogenic amino acids to glucose involves the two rate-limiting enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase (PYC). Of these two genes, only the promoter for *Pepck* was found to have a functional PPRE in mice [77]. The induction of other enzymes in this pathway is PPARα-dependent, such as glycerol-3-phosphate dehydrogenase (GPDH) and glycerol kinase (GK), as well as the aquaporins (AQP) 3 and 9 that act as liver glycerol import channels [78]. The observation that PPARα-null mice manifest lower fed and fasted glucose levels supports an involvement of PPARα in hepatic glucose production [77]. However, another report proposes as a reason for fasting hypoglycemia, the preferential channelling of glucose-6-phosphate to hepatic glycogen stores and shows unchanged glucose 6-phosphate synthesis in PPARα-null mice [79]. The pathway glycolysis/gluconeogenesis is

specifically affected by PPARα activation in mice and shows no response in human primary hepatocytes [25].

The enzyme glyoxylate reductase/hydroxypyruvate reductase (GRHPR) is important in the channelling of carbons from the glyoxylate cycle into gluconeogenesis or into the urea cycle depending on the body energy demands. In mice, PPARα activation (e.g., in the fasted state) is crucial in inducing transcriptional activation of *Grhpr*, thereby favouring a conversion of hydroxypyruvate to D-glycerate, a substrate needed in glucose synthesis [80]. In humans however, *GRHPR* expression was shown to be PPARα-independent due to promoter reorganisation during primate evolution. Moreover, alanine:glyoxylate aminotransferase (AGT), an enzyme of the glyoxylate cycle with two enzymatic activities is positively regulated by PPARα [80]. Its transaminase activity leads to the production of glycine and hydroxypyruvate.

Beyond the transcriptional activation of genes involved in lipid and glucose metabolism, the PPARα agonist WY14643 affects amino acid metabolism in rodents [81, 82]. The metabolic consequences include alterations in plasma amino acid levels. Whereas branched-chain amino acid amounts showed no change upon PPARα activation with WY14643, a significant increase in various glucogenic and some ketogenic amino acids was detected in rats [82]. Only one amino acid was lowered, namely arginine, a conditionally nonessential amino acid made in the urea cycle. mRNA levels of enzymes involved in the conversion of citrulline to arginine in the kidney are unknown, but hepatic levels of argininosuccinate synthetase (*Ass*) and argininosuccinate lyase (*Asl*) show a decrease [81, 82]. The exact mechanism of PPARα regulation of amino acid metabolism is unknown but certain genes involved in the regulation of amino acid degradation have also been shown to be negatively regulated, with the exclusion of *Grhpr* and arginase (*Arg1*) [81, 82]. The decreased amino acid degradation upon WY14643 treatment is accompanied by an increase in protein degradation. Some possible explanations for the observed amino acid mobilization upon PPARα induction are give in [82] and might be due to increased hepatic growth. The current findings are restricted to rodents and it is unclear at present if the situation is similar in humans that show no liver enlargement. One study points to a different situation in humans and describes increased plasma arginine levels after fenofibrate treatment of hypertriglyceridemic men [83]. The findings in rodents are limited to WY14643 treatment and it remains to be shown if they are of general character for PPARα ligands. The clofibrate-induced increased oxidation of branched-chain amino acids seems to be due to its direct inhibitory actions on branched-chain α-keto acid dehydrogenase kinase (BCKDK) that regulates the key enzyme of this process, and not due to effects mediated through PPARα activation [84].

Additionally, in mice, PPARα activation inhibits inflammatory gene expression by downregulation of acute phase proteins such as C-reactive protein (CRP), fibrinogen, and serum amyloid A (SAA) resulting in reduced hepatic inflammation and risk for cardiovascular disease and cancer [85]. Likewise in humans, there is a similar downregulation of plasma acute phase proteins after fenofibrate treatment [86]. Recently, it was demonstrated that the expression of the transcription factor CREBH that is exclusively found in the liver, is regulated by PPARα in both mice and humans [25]. It plays an important role in the activation of the acute inflammatory response and is also a regulator of hepatic gluconeogenesis [87, 88].

Described in mice is the reduced risk of liver damage by chemical-induced stress. Exposure to hepatotoxic agents like the environmental pollutant carbon tetrachloride (CCl_4) induces reversible liver damage [89]. The underlying reason is a decreased resistance to oxidative stress that leads to lipid peroxidation, altered calcium homeostasis, and membrane damage. Stimulated mRNA expression of uncoupling protein 2 (*Ucp2*) by PPARα in rodents results in uncoupling of the proton gradient across the inner mitochondrial membrane and a downregulation of reactive oxygen species (ROS) induced by CCl_4 metabolites [90, 91]. In addition, PPARα helps to protect from chemical-induced oxidative stress by upregulating genes of the chaperone family and of the proteasome, thereby influencing protein folding and degradation of harmed proteins in mice [92]. Furthermore, the observation that PPARα-null mice demonstrate decreased longevity, where stress response genes are of importance, and that PPARα expression decreases with age, suggests an involvement of PPARα in this process [7].

In rodents, long-term administration of PPARα leads to increased peroxisome proliferation, in addition to hepatic hypertrophy and hyperplasia that will ultimately result in liver tumors [93–98]. The carcinogenic response is based on enhanced cell replication that might increase the risk for DNA damage and altered oncogene and tumor suppressor gene expressions. Moreover, there is evidence for suppressed apoptosis in liver cells, a process important for the removal of damaged cells [99–102]. There is also a close relationship of PPARα-induced cancer formation with increased production of ROS due to peroxisome proliferation that might contribute to DNA damage [103].

Shah and colleagues have proposed changed hepatic microRNA (miR) expression via PPARα-regulation as the reason for liver cancer formation [104]. miRs are 21–23 nucleotide long sequences that are suggested to regulate the expression of up to 30% of all genes [105, 106]. Experimental evidence pointed to PPARα-involvement in several changed miR levels, in particular in the downregulation of miR let-7c by an as yet unidentified mechanism [104]. Let-7c controls c-Myc protein levels, a transcription factor regulating target genes involved in cell proliferation. Downregulation of let-7c stabilizes *c-Myc* mRNA leading to the expression of c-Myc target genes. This could be a reason for enhanced hepatocyte proliferation, that together with the induction of oxidative stress might lead to hepatocarcinogenesis in rodents. Induction of hepatocarcinogenesis seems to be restricted to rodents and is not documented in humans (extensively reviewed in [107]). Cancer formation after PPARα activation in tissues other than the liver has been described in rats and includes testicular (Ledig cell) and pancreatic acinar cell tumors [108]. However, if these findings are of significance for humans requires further in-depth risk assessments.

In summary, the hepatic response to PPARα activation is essential under fasting conditions. PPARα activation by FAs released from the adipose tissue leads to induction of several metabolic processes in mice: β-oxidation, ketogenesis, glycolysis/gluconeogenesis, with concomitant reduction of amino acid catabolism and an anti-inflammatory response. The changes result in an increased plasma concentration of glucose and ketone bodies and decreased urea and acute phase proteins. PPARα is important in both mice and humans for the regulation of lipid metabolism. In contrast to mice, humans show no effect on the glycolysis/gluconeogenesis pathway. One pathway specifically affected in humans and not in mice is apolipoprotein production. In humans treated with a PPARα activator, hepatic transcription activation leads to decreased VLDL production and plasma TAG levels, but increased HDL cholesterol, important parameters in the treatment for dyslipidemia, type 2 diabetes, or cardiometabolic disorders.

3. PPARα in Skeletal Muscle

In human skeletal muscles, three main muscle fiber types, type I (oxidative, slow twitch), IIA (intermediate) and IIX (glycolytic, fast twitch), can be delineated based on histochemical, functional and biochemical properties (reviewed in [109]). In human skeletal muscle cells *in vitro*, *PPARα* was shown to be induced early during myocyte differentiation [110, 111]. A correlation between the expression of *PPARα*, proportion of type I fibers and endurance exercise has been found in human skeletal muscle *in vivo* [112, 113]. The expression of *PPARα* (as well as of *PPARδ* and the *PPARγ* coactivator (*PGC*)-1*α* and -*1ß*) in skeletal muscle was increased in athletes and reduced in spinal cord-injured subjects [113]. The observed increase of *PPARα* expression after endurance training [112, 114] was greater in type I fibers than in type IIA and IIX fibers [112]. Also in rat skeletal muscle, fiber-type specific PPARα activation was found. When treated with the PPARα agonist fenofibrate, 26 genes were identified that were significantly regulated in soleus (type I) but not in quadriceps femoris (type II) rat muscle [115]. The correlation of *PPARα* expression and exercise has not been found in animal studies. In rats, four weeks of exercise did not change the *PPARα* mRNA expression in skeletal muscle in control chow-fed animals, and in fat-fed rats exercise counteracted the diet-induced increase of *PPARα* expression [116].

Both in human and rodent skeletal muscle, activation of PPARα affects lipid metabolism. Activation of PPARα by a potent agonist (GW7647) in differentiated human myotubes *in vitro* stimulated lipid oxidation [110, 117] and decreased accumulation of TAG [110]. Other, less potent PPARα agonists did not increase lipid oxidation in human myotubes [118]. In the same cell model, GW7647 upregulated the expression of pyruvate dehydrogenase kinase (*PDK*)4 [119]. PDK4 is an important isoenzyme regulating the activity of pyruvate dehydrogenase complex. The enzyme phosphorylates and inhibits the pyruvate dehydrogenase complex and thereby blocks the entry of carbohydrates into the mitochondria for oxidation (for reviews see [120, 121]. *Pdk4* was also induced in rat gastrocnemius muscle after treatment of the animals with the PPARα agonist WY14643, by streptozotocin-induced diabetes, or by starvation, i.e. conditions where increased levels of long-chain fatty acids may activate PPARα [122]. Pathway analysis of the genes significantly regulated in soleus (type I), but not in quadriceps femoris (type II) muscle by fenofibrate in rats, revealed that the most significant function represented in the gene set was lipid metabolism [115]. Treatment with a potent PPARα agonist increased the expression of *Cpt-1* in hamster soleus muscle [123].

Influence of PPARα on both lipid and glucose metabolism was highlighted in transgenic mice overexpressing PPARα in skeletal muscle [124]. In these animals many known PPARα target genes involved in cellular fatty acid import and binding, TAG synthesis, and mitochondrial and peroxisomal β-oxidation were activated, and genes involved in cellular glucose utilization were downregulated in skeletal muscle. Basal and insulin-stimulated glucose uptake was reduced in isolated skeletal muscle, and the transgenic animals developed glucose intolerance despite being protected from diet-induced obesity [124]. In contrast, in PPARα-null mice, glucose tolerance, insulin-stimulated glucose disposal and glucose uptake were increased in spite of high fat-induced weight gain and increased levels of TAGs in muscle [124]. In another study, fatty acid oxidation in skeletal muscle was found to be reduced by 28% in starved PPARα-null mice compared to wild type (WT) mice, however in fed animals fatty acid oxidation in PPARα-null and WT mice was similar [125]. TCA cycle intermediates, amino acids and short-chain acylcarnitine species were reduced in skeletal muscle of PPARα-null mice compared to WT mice, indicating impaired TCA cycle flux and increased protein catabolism combined with defects in fatty acid catabolism in PPARα-null mice [37].

In humans and mice, a negative side effect of PPARα activation in muscle is in rare cases (<1%) muscle weakness and pain (myopathy) or very seldom breakdown of muscle (rhabdomyolysis) [126–129]. In particular, type I fibers are affected by skeletal muscle toxicity in rats [115]. The exact mechanisms are unclear at present, but might include oxidative stress and tissue damage from elevated peroxisomal and mitochondrial β-oxidation [130].

PPARα also seems to exert a role in protecting against ischemic injury in skeletal muscle as well as in heart and liver [131]. Thus, in mouse skeletal muscle, loss of the oxygen sensor prolyl oxidase (PHD)1 was found to lower oxygen consumption by shifting to a more anaerobic glucose utilization through activation of PPARα-dependent genes [131].

Another PPAR, PPARδ, is the most abundant PPAR isoform in skeletal muscle. Similar to *PPARα*, the expression of *PPARδ* has been described to be higher in type I fibers compared to type II fibers (reviewed in [132]). Also alike to PPARα, activation of PPARδ induces a number of genes involved in fatty acid import and oxidation, and increases lipid oxidation in skeletal muscle [125, 133–136], indicating redundancy in the functions of PPARα and δ as regulators of fatty acid metabolism [125]. However, in contrast to PPARα, activation of PPARδ has been shown to increase glucose

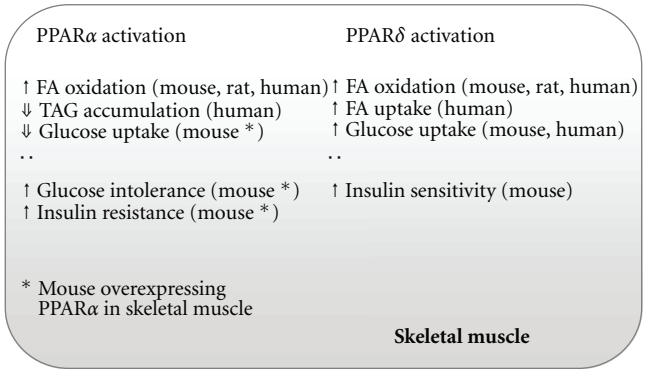

Figure 2: Examples of metabolic effects of PPARα or PPARδ activation in skeletal muscle. FA, fatty acid; TAG, triacylglycerol. For references, see the text.

uptake [136, 137] and prevent insulin resistance in skeletal muscle (Figure 2) [138].

In summary, PPARα has been shown to be involved in lipid and glucose metabolism in skeletal muscle. PPARα activation increases lipid oxidation and decreases TAG accumulation. Overexpression of *PPARα* in skeletal muscle causes reduced glucose uptake in muscle and glucose intolerance in the animals, while PPARα-null mice show increased glucose tolerance, increased insulin-stimulated glucose disposal and enhanced glucose uptake in skeletal muscle, in spite of high fat-induced weight gain and increased levels of TAGs in muscle. Thus, PPARα activation may potentially exert both beneficial and undesirable effects on skeletal muscle fuel metabolism. Activation of PPARα and PPARδ seems to have overlapping effects on fatty acid metabolism, but possibly different effects on glucose metabolism in skeletal muscle.

4. Concluding Remarks

The transcription factor PPARα influences metabolism through activation of many target genes in a variety of metabolically active tissues, in particular under fasting conditions. Cross-species prognostics are not always possible due to differences in metabolism, expression levels, or diet. While observations in rodents could have pointed to risks for human treatment with PPARα agonists (e.g., hepatocarcinogenesis, skeletal muscle insulin resistance, and myopathy) it has been shown that in humans, PPARα activation is a useful therapeutic target in treating metabolic disorders. Clinical studies on drug-induced PPARα activation include fibrates, statins, and more recently the combination of statins with fibrates. In humans, fibrates have the characteristic of reducing TAG levels and increasing HDL cholesterol, however not all trials show a vascular benefit. In some trials, clinical end-points like the rate of coronary heart disease in type 2 diabetes patients (VAHIT: Veterans affairs HDL intervention Trial, [139]) or the progression of atherosclerosis in young men after a first myocardial infarction (BECAIT: Bezafibrate Coronary Atherosclerosis Intervention Trial, [140]) could be reduced by treatment. Statin therapy shows more consistent benefits with decreased plasma LDL cholesterol levels and reduced vascular disorders and death [141]. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) lipid study, addressed whether a fibrate (fenofibrate) and statin (simvastatin) combination would reduce the rate of cardiovascular events more than individual treatments in type 2 diabetes patients [142]. The combination treatment however did not influence the primary outcome significantly more than simvastatin alone, but instead showed a sex-dependent difference, with more benefits for men than women.

Rodent studies are mostly done in male animals, but the response of PPARα activation in male versus females was investigated in some studies and seems to be influenced by estrogen [143, 144]. This female hormone inhibits PPARα action and represses lipid regulatory pathways in the liver. Thus, in the treatment with PPARα agonists, gender-differences have to be taken into consideration and while therapy might be advantageous against lipid disorders in men and postmenopausal women with no interfering estrogen, premenopausal women might not benefit from the same treatment [145].

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Review Article

PPARα in Obesity: Sex Difference and Estrogen Involvement

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Peroxisome proliferator-activated receptor α (PPARα) is a member of the steroid hormone receptor superfamily and is well known to act as the molecular target for lipid-lowering drugs of the fibrate family. At the molecular level, PPARα regulates the transcription of a number of genes critical for lipid and lipoprotein metabolism. PPARα activators are further shown to reduce body weight gain and adiposity, at least in part, due to the increase of hepatic fatty acid oxidation and the decrease in levels of circulating triglycerides responsible for adipose cell hypertrophy and hyperplasia. However, these effects of the PPARα ligand fenofibrate on obesity are regulated with sexual dimorphism and seem to be influenced by the presence of functioning ovaries, suggesting the involvement of ovarian steroids in the control of obesity by PPARα. In female ovariectomized mice, 17β-estradiol inhibits the actions of fenofibrate on obesity through its suppressive effects on the expression of PPARα target genes, and these processes may be mediated by inhibiting the coactivator recruitment of PPARα. Thus, it is likely that PPARα functions on obesity may be enhanced in estrogen-deficient states.

1. Introduction

Obesity is the result of an energy imbalance caused by an increased ratio of caloric intake to energy expenditure. In conjunction with obesity, related metabolic disorders such as dyslipidemia, atherosclerosis, and type 2 diabetes have become global health problems. The peroxisome proliferator-activated receptors (PPARs) have been the subject of intense investigation and considerable pharmacological research due to the fact that they are involved in the improvement of these chronic diseases. Three PPAR isotypes have been identified: PPARα, PPARγ, and PPARβ/δ, each with different ligand specificity, very distinct tissue distributions, and different biological functions.

Among the three subtypes, PPARα is expressed predominantly in tissues that have a high level of fatty acid (FA) catabolism such as liver, heart, and muscle [1–3]. PPARα regulates the expression of a large number of genes that affect lipid and lipoprotein metabolism [4–7]. PPARα ligands fibrates have been used for the treatment of dyslipidemia due to their ability to lower plasma triglyceride levels and elevate HDL cholesterol levels. PPARα is also thought to be involved in energy metabolism. Since PPARα ligands fibrates stimulate hepatic FA oxidation and thus reduce the levels of plasma triglycerides responsible for adipose cell hypertrophy and hyperplasia, PPARα may be important in the control of adiposity and body weight due to its ability to regulate an overall energy balance. This notion is supported by findings showing that PPARα-deficient mice exhibited abnormalities in triglyceride and cholesterol metabolism and became obese with age [8]. Furthermore, several studies have suggested that fibrates can modulate body weight and adiposity in experimental animal models, such as fatty Zucker rats, high fat-fed C57BL/6 mice, and high fat-fed obese rats [9–11].

Energy balance seems to be influenced by gonadal sex steroids [12]. Female sex steroid hormones have been the subject of intense investigation over the last several decades based on the role that these ovarian hormones play in regulating food intake, body weight, and lipid metabolism. For example, ovariectomized (OVX) animals and postmenopausal women show increased food intake, body weight, and adipose tissue mass, as well as decreased FA oxidation and triglyceride lipolysis, indicating the involvement of gonadal steroids in the modulation of obesity [13–16]. Several lines of study show that ovarian steroids, in particular estrogens, can affect obesity and the related disorders

of dyslipidemia, type 2 diabetes, and cardiovascular disease (CVD) [12]. Estrogen insufficiency is known to be largely responsible for increased adiposity and circulating lipids in OVX rodents because such animals do not display obesity, adiposity, and lipid disorders when they are administered exogenous estrogens [17–19]. Moreover, my previous results demonstrated that fenofibrate reduced body weight and white adipose tissue (WAT) mass in male and female OVX mice [20–23]. Although the administration of 17β-estradiol (E2) or fenofibrate alone effectively reduces body weight gain and WAT mass in female OVX mice, fenofibrate treatment does not prevent gains in body weight and WAT mass in the presence of ovaries. Interestingly, there are data indicating that PPAR/RXR heterodimers are capable of binding to estrogen response elements (EREs), and PPAR and estrogen receptors (ERs) share cofactors [24–28], suggesting that signal cross-talk may exist between PPARα and ERs in the control of obesity.

Based on my published results showing the fenofibrate functions on obesity during various conditions, this paper will focus on the differential regulation of PPARα on obesity by sex differences and the interaction of PPARα and ERs in the regulation of obesity.

2. General Aspects of PPARα and ERs

2.1. PPARα and ERs as Nuclear Hormone Receptors. Both PPARα and ERs belong to the nuclear hormone receptor superfamily, which has a typical structure consisting of six functional domains, A/B, C, D, and E/F (Figure 1) [29–31]. The amino-terminal A/B domain contains a ligand-independent activation function-1 (AF-1). The C or DNA binding domain (DBD) contains the structure of the two zinc fingers and α-helical DNA motifs. The DBD directs nuclear receptors to the hormone response elements (HREs) of target genes. The D region is a highly flexible hinge region and may be involved in protein-protein interactions, such as receptor dimerization and efficient binding of DBD to HREs. The E/F domain is responsible for ligand-binding and is thus named the ligand binding domain (LBD). The interaction of nuclear receptors with their ligands induces conformational changes that include the AF-2 ligand-dependent activation domain, which is located in the C-terminal α-helix. AF-2 regulates ligand-dependent transactivation, recruitment of coactivators, and release of corepressors. In addition, AF-2 is also important for receptor dimerization.

Molecular signaling of PPARα and ERs functions is similar [34–37]. In the unliganded or antagonist-bound state, they are associated with corepressor proteins such as nuclear receptor corepressor (NCoR) or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Figure 2(a)). After binding within the LBD, PPARα ligands induce heterodimerization with retinoid X receptor (RXR) and the subsequent interaction with coactivators like CREB-binding protein (CBP) or steroid receptor coactivators, followed by binding to PPAR response elements (PPREs) within target gene promoters (Figure 2(b)). Similarly, ligand-activated ERs bind to their half-site-containing EREs as homodimers following the recruitment of coactivators. Importantly, PPARα shares a similar pool of cofactors with ERs which provides a basis for mutual interactions between these receptors [34, 35].

2.2. PPARα. PPARα was the first PPAR to be identified by Issemann and Green in 1990, and human PPARα was cloned by Sher et al. in 1993 [1, 38]. PPARα is predominantly expressed in tissues with high rates for mitochondrial and peroxisomal FA catabolism such as liver, brown adipose tissue (BAT), heart, skeletal muscle, kidney, and intestinal mucosa [1–3]. Significant amounts of PPARα are present in different immunological and vascular wall cell types [39, 40].

PPARα acts as a ligand-activated transcription factor. PPARα mediates the physiological and pharmacological signaling of synthetic or endogenous PPARα ligands. FAs and FA-derived compounds are natural ligands for PPARα. Modified FAs, conjugated FAs, oxidized phospholipids, and FA-derived eicosanoids such as 8-*S*-hydroxytetraenoic acid and leukotriene B4 activate PPARα [41]. Synthetic compounds can also activate PPARα. These compounds include carbaprostacyclin, nonsteroidal anti-inflammatory drugs, pirinixic acid (also known as Wy14,643), phthalate ester plasticizers, and hypolipidemic drugs fibrates [41]. Of the currently used fibrates, fenofibrate, gemfibrozil, clofibrate, and ciprofibrate preferentially activate PPARα whereas bezafibrate activates all three PPARs. Novel PPARα/γ dual agonists and PPAR$\alpha/\gamma/\delta$ pan agonists with PPAR selective modulator activity are under development as drug candidates [42, 43].

PPARα regulates the expression of a number of genes critical for lipid and lipoprotein metabolism, thereby leading to lipid homeostasis. Ligand-bound PPARα heterodimerizes with RXR and binds to direct repeat PPREs in the promoter region of target genes (Figure 3(a)). PPARα target genes include those involved in the hydrolysis of plasma triglycerides, FA uptake and binding, and FA β–oxidation (Table 1). Genes involved in the HDL metabolism are also regulated by PPARα. The activation of PPARα target genes therefore promotes increased β-oxidation of FAs, as well as the decrease in high circulating triglyceride levels and increased high HDL cholesterol levels, leading to lipid homeostasis.

In addition to PPARα regulation of genes for lipid and lipoprotein metabolism, PPARα regulates the expression of uncoupling proteins (UCPs), which contain PPRE in their promoters. PPARα activators increase the mRNA levels of UCP1 in BAT, UCP2 in liver, and UCP3 in skeletal muscle. UCP1 regulates energy expenditure through thermogenesis. Reductions in body weight and adiposity by fenofibrate are associated with elevation of hepatic UCP2 expression [44]. Transgenic mice overexpressing UCP3 in their skeletal muscle exhibit increased FA oxidation and are resistant to diet-induced obesity. Thus, PPARα may be involved in energy balance and obesity by regulating UCPs [45].

In addition to the important roles of PPARα in FA oxidation in liver and skeletal muscle, PPARα activators may affect adipose tissue metabolism. For example, administration of bezafibrate, a typical PPAR activator, leads to dedifferentiation of adipocytes into preadipocyte-like cells through

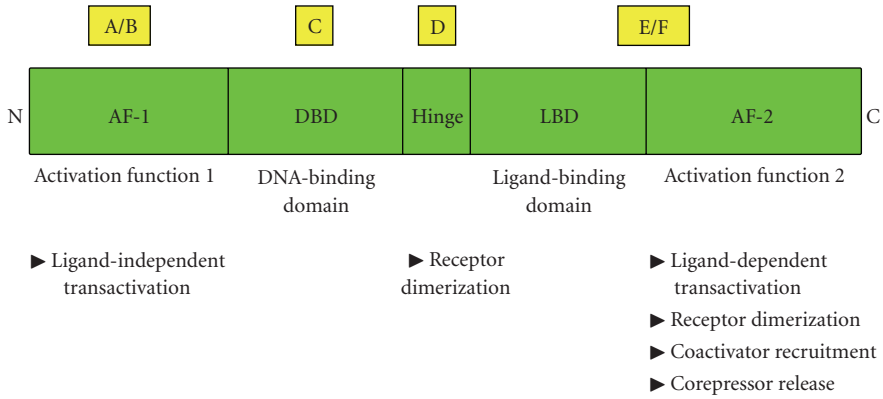


Figure 1: *Schematic structure of the functional domains of nuclear receptors.* The activation domains AF-1 and AF-2 are located at the N-terminal and C-terminal regions, respectively. C domain is a highly conserved DNA-binding domain. D domain is a highly flexible hinge region. E/E domain is responsible for ligand-binding and converting nuclear receptors to active forms that bind DNA. Adapted from [29].

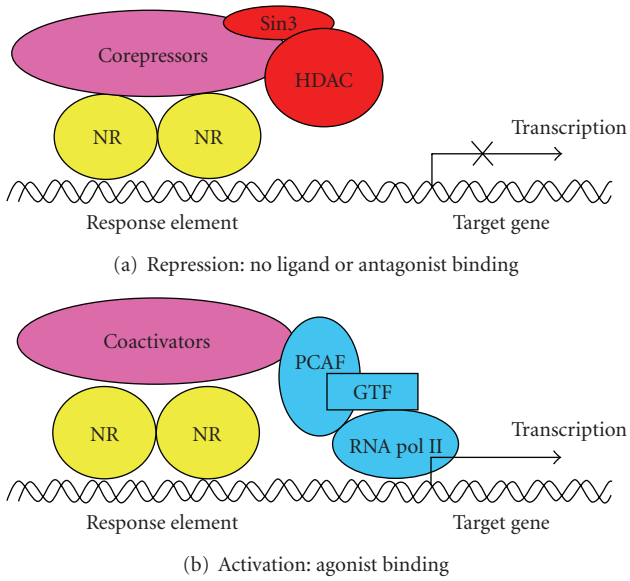


Figure 2: *Activation and repression of nuclear receptor activity.* (a) In the absence of ligand, nuclear receptors (NRs) are associated with corepressor complexes that bind Sin3 and histone deacetylase (HDAC), thereby turning off gene transcription. Some steroid receptors can recruit this complex when they are occupied by antagonists although they do not seem to be associated with corepressors in the unliganded state. (b) In the presence of ligand, NRs generally recruit coactivator complexes, PCAF histone acetyltransferase protein, general transcription factors, and RNA polymerase II to induce gene transcription. GTF: general transcription factor; RNA pol II: RNA polymerase II; PCAF: P300/CBP-associated factor.

the activation of genes involved in both mitochondrial and peroxisomal β-oxidation [46]. The PPARα ligand GI259578A decreases the mean size of adipocytes in WAT [47]. This is supported by my recent report that fenofibrate stimulates FA β–oxidation in both epididymal adipose tissue and differentiated 3T3-L1 adipocytes [48].

Table 1: PPARα target genes involved in lipid homeostasis.

Target genes	Gene expression
Fatty acid uptake, binding, and activation	
Fatty acid transport protein (FATP)	Stimulation
Fatty acid translocase (FAT/CD36)	Stimulation
Liver cytosolic fatty acid-binding protein (L-FABP)	Stimulation
Acyl-CoA synthetase (ACS)	Stimulation
Carnitine palmitoyltransferase I and II (CPT-1and CPT-II)	Stimulation
Mitochondrial fatty acid β-oxidation	
Very long-chain acyl-CoA dehydrogenase (VLCAD)	Stimulation
Long chain acyl-CoA dehydrogenase (LCAD)	Stimulation
Medium-chain acyl-CoA dehydrogenase (MCAD)	Stimulation
Short-chain acyl-CoA dehydrogenase (SCAD)	Stimulation
Peroxisomal fatty acid β-oxidation	
Acyl-CoA oxidase (ACOX)	Stimulation
Bifunctional enzyme (HD)	Stimulation
3-Ketoacyl-CoA thiolase (Thiolase)	Stimulation
Hydrolysis of plasma triglycerides	
lipoprotein lipase (LPL)	Stimulation
Apolipoprotein C-III (Apo C-III)	Inhibition
Fatty acid synthesis	
Acetyl-CoA carboxylase (ACC)	Inhibition
Fatty acid synthase (FAS)	Inhibition
HDL metabolism	
Apolipoprotein A-I and A-II (ApoA-I and ApoA-II)	Stimulation
ATP-binding cassette transporter 1 (ABCA1)	Stimulation
Electron transport chain	
Uncoupling protein 1, 2, and 3 (UCP1, 2, and 3)	Stimulation

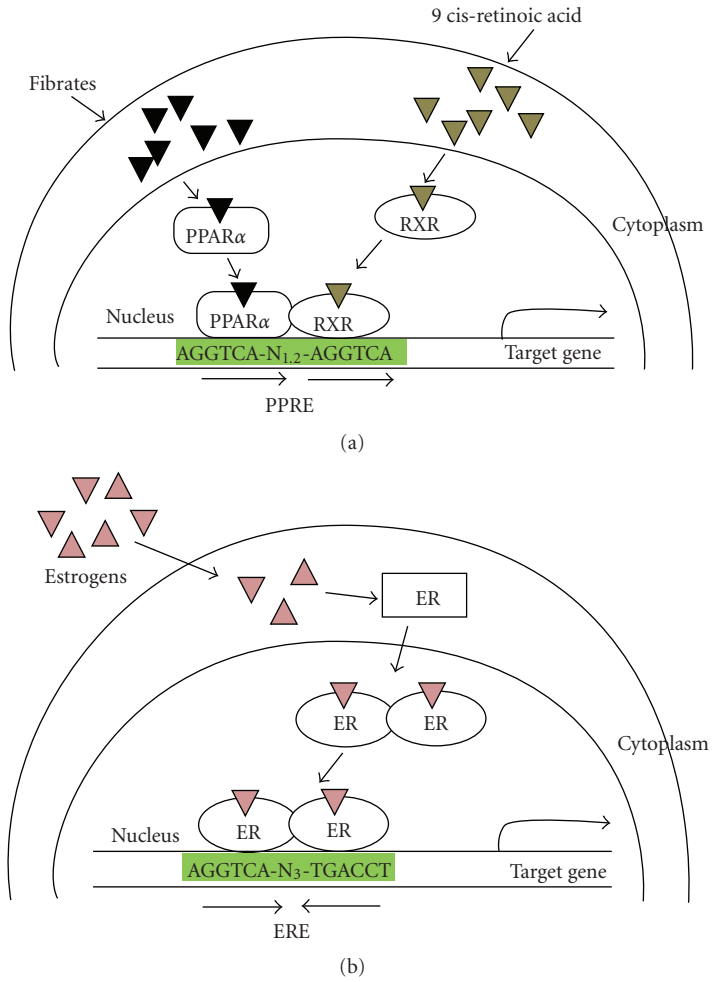


Figure 3: *The signaling pathways of PPARα and estrogen receptors.* (a) After activation by its respective ligands, PPARα heterodimerizes with retinoid X receptor and binds to direct repeat PPRE in the promoters of target genes to drive expression of target genes. (b) Estrogen-bound estrogen receptors recognize palindromic ERE to directly bind this DNA and ultimately increase gene expression. RXR: retinoid X receptor; PPRE: PPAR response element; ERE: estrogen response element; ERs: estrogen receptors.

PPARα may be involved in the regulation of energy balance through fat catabolism. Since fenofibrate increases hepatic FA oxidation and thus decreases the levels of plasma triglycerides responsible for adipose cell hypertrophy and hyperplasia, it may inhibit an increase in body weight. This is supported by a report that PPARα-deficient mice showed abnormal triglyceride and cholesterol metabolism and became obese with age [8]. Expression of PPARα and FA oxidative PPARα target genes is suppressed in obese mice [49]. Many studies show that fenofibrate can modulate body weight in animal models of diabetes, obesity, and insulin resistance although another known PPARα stimulator perfluorooctanoic acid induces overweight at low doses in intact female mice [9–11, 50].

PPARα also regulates insulin resistance and diabetes due to visceral obesity. Fenofibrate prevents adipocyte hypertrophy and insulin resistance by increasing FA β-oxidation and intracellular lipolysis from visceral adipose tissue, showing that PPARα may be one of the major factors leading to decreased adipocyte size and improved insulin sensitivity [48]. Moreover, PPARα agonist treatment has been reported to improve pancreatic β-cell function in insulin-resistant rodents and the adaptive response of the pancreatic β-cell function to pathological conditions, such as obesity [51, 52]. In addition, PPARα agonists, including fibrates, normalize atherogenic lipid profile, as well as several cardiovascular risk markers [53].

2.3. ERs. Like PPARα, ERs function as ligand-dependent transcription factors belonging to members of the nuclear hormone receptor family. Two major ERs (ERα and ERβ) mediate the physiological and pharmacological signals of natural or synthetic ER activators. Upon estrogen binding, ERs are activated and act as transcriptional modulators by binding to palindromic EREs in the promoter region of target genes (Figure 3(b)) [54, 55]. ERs are also activated by specific synthetic ligands such as raloxifene, tamoxifen, and the ERβ-specific ligand diarylpropionitrile. ERα is mainly expressed in the female reproductive system such as ovary, uterus, pituitary, and mammary glands but is also present in

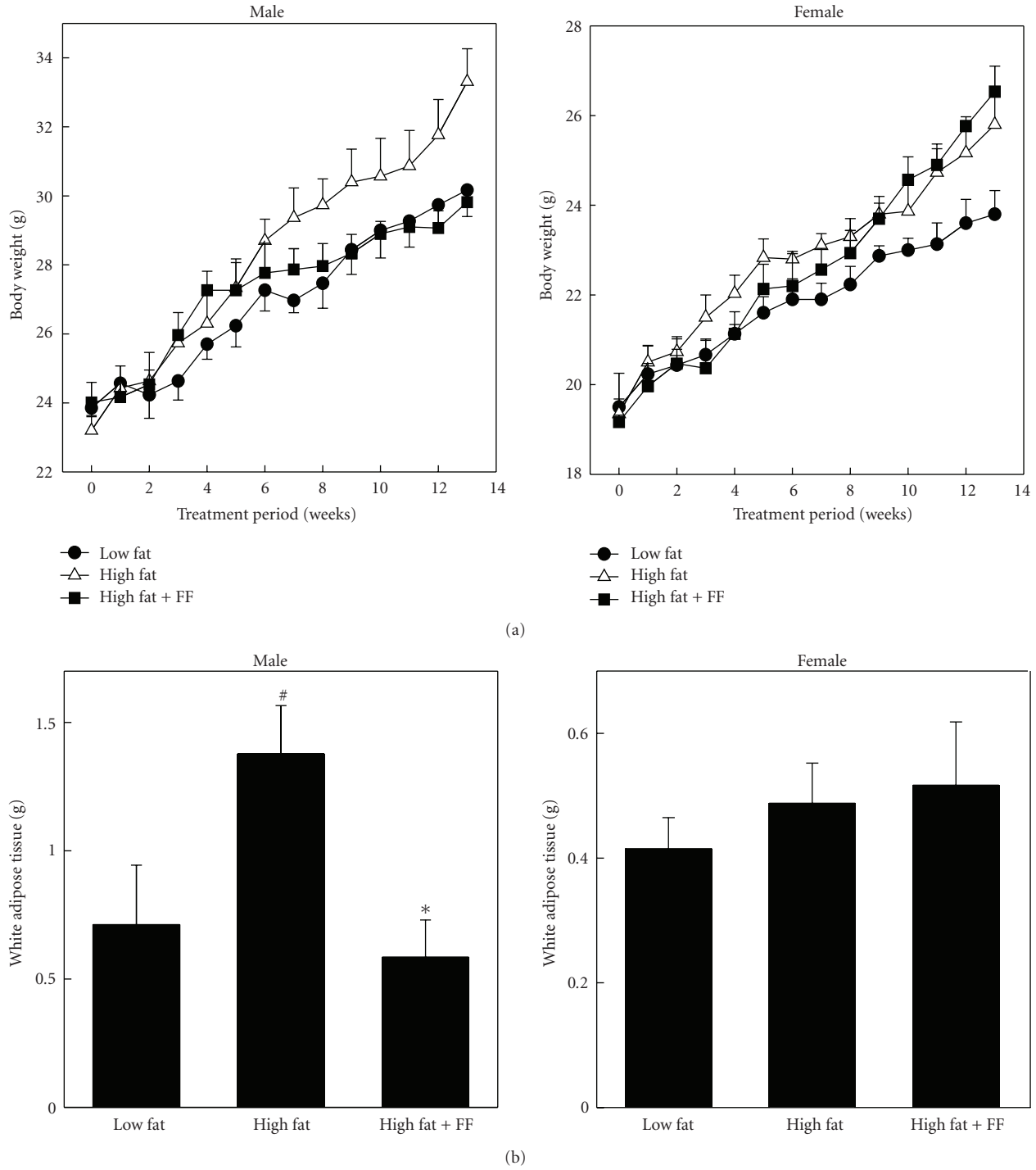


Figure 4: *Effects of fenofibrate on high fat diet-induced body weight gain (a) and WAT mass (b) in both sexes of C57BL/6 mice.* Male and female C57BL/6 mice were received a low fat, high fat, or high fat diet supplemented with fenofibrate (0.05% w/w) for 13 weeks. Body weight at the end of the experiment are statistically different ($P < .01$) between high fat diet and high fat plus fenofibrate groups. # : Significantly different versus a low fat diet group, $P < .05$. $*$: Significantly different versus a high fat diet group, $P < .01$. Adapted from [20].

the hypothalamus, brain, bone, liver, WAT, skeletal muscle, and the cardiovascular system [56–58]. ERβ is expressed in many tissues including skeletal muscle, WAT, BAT, prostate, salivary glands, testis, ovary, vascular endothelium, the immune system, and certain neurons of the central and peripheral nervous system [59, 60].

The natural forms of estrogens are E2, estrone, and estriol. E2 potently activates ER-mediated transcriptional

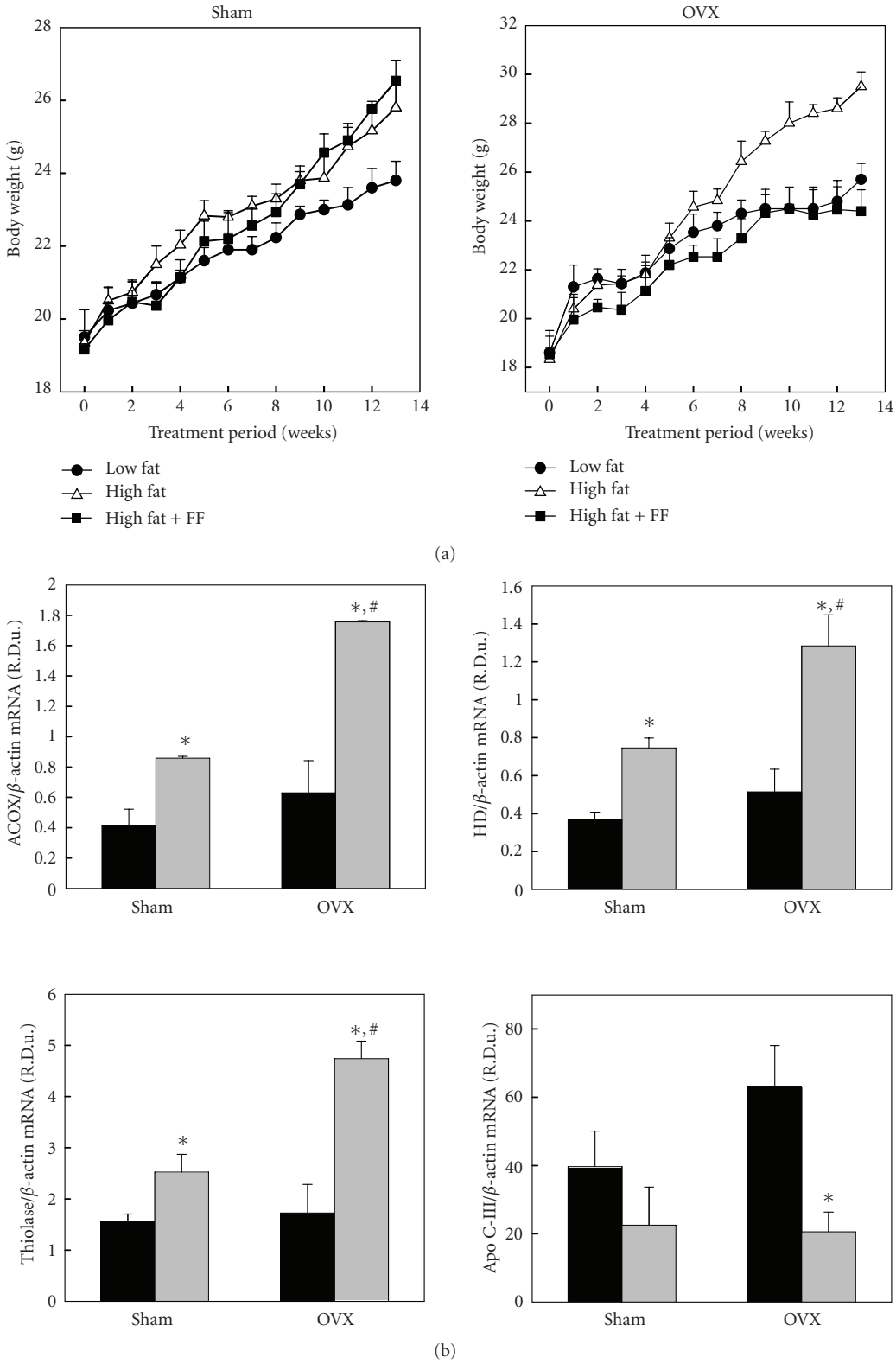


Figure 5: *Differential regulation of body weight gain (a) and PPAR α target gene expression (b) by fenofibrate depending on the presence of ovaries.* Female sham-operated (Sham) and ovariectomized (OVX) mice received a low fat, high fat, or fenofibrate-supplemented (FF; 0.05% w/w) high fat diet for 13 weeks. Body weights at the end of the treatment period are significantly different not only when comparing the low fat group to either the high fat ($P < .05$) or high fat plus FF ($P < .01$) groups in female Sham mice, but also when comparing the high fat group to either the low fat ($P < .01$) or high fat plus FF ($P < .005$) groups in female OVX mice. $*$: Significantly different versus the high fat group, $P < .05$. # : Significantly different versus the Sham group, $P < .05$. ACOX: acyl-CoA oxidase; HD: enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; thiolase: 3-ketoacyl-CoA thiolase; apo C-III: apolipoprotein C-III. Adapted from [23].

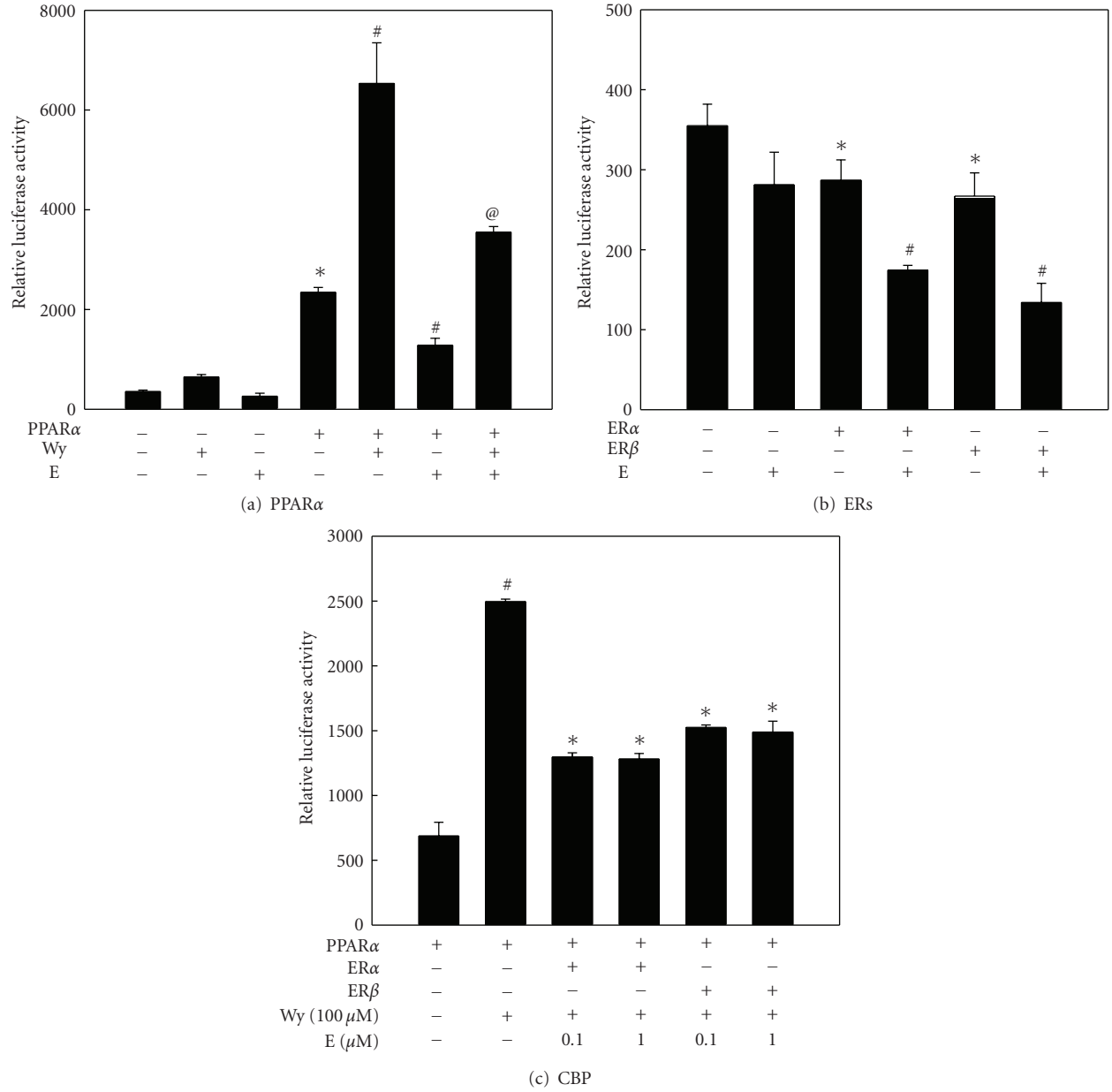
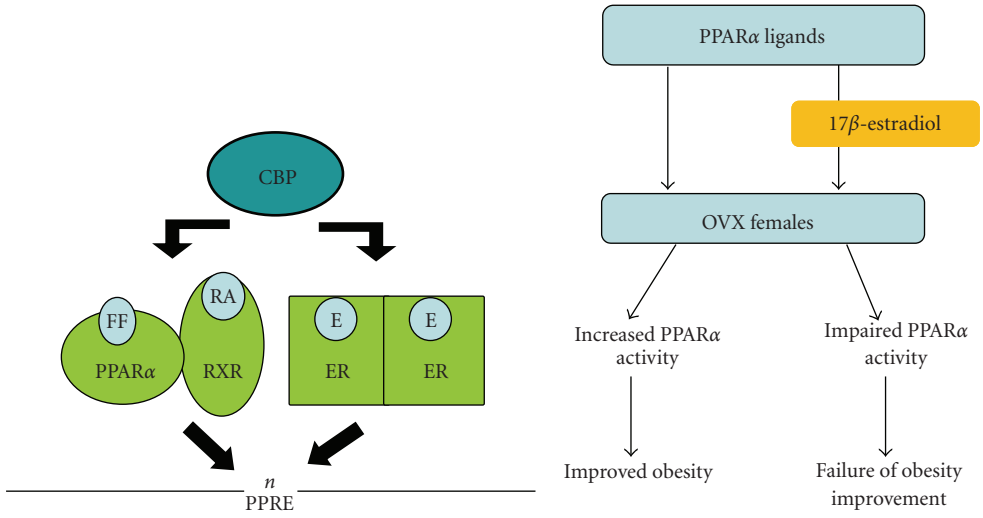


Figure 6: *Inhibition of PPARα reporter gene expression ((a) and (b)) and coactivator recruitment (c) by 17 β-estradiol.* (a) NMu2Li cells were transiently transfected with expression plasmids for PPARα and $PPRE_3$-TK-Luc reporter. * Significantly different versus control group, $P < .0001$. # : Significantly different versus PPARα group $P < .0001$. @ Significantly different versus PPARα/Wy group, $P < .001$. (b) NMu2Li cells were transiently transfected with expression plasmids for $PPRE_3$-TK-Luc reporter and ERα or ERβ.∗: Significantly different versus control group, $P < .05$.# : Significantly different versus respective ER group, $P < .01$. (c) CV-1 cells were transiently transfected with expression plasmids for VP16-mPPARα, GAL-CBP, reporter plasmid pFR-Luc, and VP16-hERα or VP16-hERβ. #: Significantly different versus PPARα group, $P < .01$.∗ : Significantly different versus PPARα/Wy group, $P < .005$. Adapted from [32].

activity to a greater extent than estrone or estriol. E2 has been considered one of the most important hormones in female physiology and reproduction for a long period. However, we now know that E2 also plays a protective role in a variety of pathophysiological states, such as obesity, cardiovascular disease, hyperlipidemia, diabetes, osteoporosis, and cancer in both men and women [61].

E2 is involved in the regulation of adiposity and obesity, and visceral fat varies inversely with E2 levels [62]. Accumulation of visceral fat occurs in females when E2 levels become sufficiently low. In rodents, ovariectomy leads to weight gain primarily in the form of adipose tissue, which is reversed by physiologic E2 replacement [12, 63–65]. Loss of circulating E2 is associated with an increase in adiposity during menopause whereas postmenopausal



(a) Competition for coactivators (b) Differential regulation of obesity

FIGURE 7: *Mechanism of inhibitory effect of 17β-estradiol on PPAR α-mediated regulation of obesity.* (a) Competition between PPARα and estrogen receptors (ERs) for coactivator binding. 17β-estradiol-activated ERs can interfere with the PPRE binding of PPARα. (b) Inhibition of PPARα actions on obesity by E**.** E impairs the ability of PPARα ligands to reduce body weight gain and adiposity in female ovariectomized (OVX) mice. FF: fenofibrate; RA: 9 cis-retinoic acid; RXR: retinoid X receptor. Adapted from [33].

women who receive E2 replacement therapy do not display the characteristic abdominal weight gain pattern usually associated with menopause [13–15]. Aromatase deficiency, during which E2 is not produced, results in the development of adiposity and obesity [66]. Furthermore, ERα deficiency increased adipose tissue in both male and female mice, consistent with other reports linking estrogen with body weight regulation and adipocyte function [67]. E2 influences food intake and eventually the maintenance of normal body weight in adult females. In female dogs, a phasic decrease in food intake occurs during estrus [68]. Gradual decreases in eating through the follicular phase have been shown in monkeys, which show progressive increases in estrogens through the follicular phase comparable to those of humans [69]. E2 treatment to OVX rats normalized meal size, food intake, and body weight gain to the levels observed in intact rats [19, 70]. ERβ is involved in the anorectic action of E2. Blockade of ERβ inhibits the effects of E2 on food intake, body weight gain, and fat accumulation in OVX rats [71]. In contrast, Heine et al. [67] and D'Eon et al. [16] suggested that E2 decreases adiposity and adipocyte size in OVX mice independent of differences in energy intake, possibly through promoting fat oxidation and enhancing triglyceride breakdown [16, 67].

In addition to food intake and body weight regulation, estrogen improves glucose homeostasis and diabetes mellitus. Mice that lack ERα have insulin resistance and impaired glucose tolerance [67]. Both male and female aromatase-KO mice have reduced glucose oxidation, and male aromatase-KO mice develop glucose and insulin resistance that can be reversed by E2 treatment [58, 66]. ERα and ERβ modulate glucose transporter 4 expression and stimulate glucose uptake in skeletal muscle of mice [58]. Estrogens have also been shown to regulate vascular disease. Premenopausal women have a lower tendency to develop hypertension than do men of similar age, but the prevalence of CVD increases more rapidly in aging women than in men [72]. The increased incidence of CVD in aged women may be due to the development of obesity. Although the rate of increase of CVD is greater at the postmenopausal age in women than at the same age in men, the actual incidence of CVD is still less in women than in men if hypertension is not included (Framington Heart Study). Thus, estrogen signaling through ERs leads to improvement of metabolic disorders.

As mentioned above, both PPARα and ERs have similar structures, action mechanisms, and functions, suggesting the interaction of PPARα with ERs in the control of these metabolic diseases including obesity. However, signal cross-talk between PPARα and ERs in the regulation of obesity is not clear.

3. PPARα Functions on Obesity

Over the last several decades, a number of studies have been published on the physiology, pharmacology, and functional genomics of PPARα. In vivo and in vitro studies demonstrate that PPARα plays a central role in lipid and lipoprotein metabolism, and thereby decreases dyslipidemia associated with metabolic syndrome. Obesity is the leading cause for the development of metabolic diseases, such as obesity, type 2 diabetes, dyslipidemia, and CVD. There are important sex differences in the prevalence of obesity-related metabolic diseases [33, 73–75]. Ovarian hormones seem to have protective roles in metabolic diseases since women with functioning ovaries have much fewer incidences of such disorders, but these metabolic diseases dramatically increase in postmenopausal women.

3.1. Fenofibrate Regulates Obesity with Sexual Dimorphism. PPARα activator fenofibrate differentially influences body weight and adiposity in both sexes of mice. Fenofibrate improves body weight gain and adiposity in high fat-diet-fed male mice, but fails to regulate them in female mice (Figure 4) [20]. In males, body weight and WAT mass increased by 44% and 77%, respectively, after 14-week administration of high fat diet. These parameters were lowered after fenofibrate treatment, more so than those of mice given a low fat diet, and the reduction in body weight correlated with a fall in adipose tissue mass. In contrast to males, fenofibrate slightly increased high fat diet-induced body weight and adipose tissue mass in female mice, suggesting a different PPARα action on females than on males in the control of obesity. Previous studies showed that fenofibrate can modulate body weight and adiposity in several animal models [9–11]. Since these results were obtained from males, fenofibrate may be an effective regulator of energy homeostasis in the male animal system. Taken together, these studies show that body weight gain and adipose tissue mass of male C57BL/6 mice were significantly reduced by fenofibrate, but those of females were not, and indicate that the action of fenofibrate on body weight and adiposity is different, depending on sex.

Although fibrates are drugs widely used to lower elevated plasma triglycerides and cholesterol, fenofibrate is shown to control lipid metabolism with sexual dimorphism. Serum concentrations of total cholesterol and triglycerides were significantly reduced by fenofibrate in male mice, similar to the previous reports [76, 77]. However, fenofibrate not only failed to decrease total cholesterol, but also decreased circulating level of triglycerides in female mice to a much lower extent than in similarly treated males. Based on the information that lipids accumulated in the adipose tissue are largely derived from circulating triglycerides, differential regulation of adiposity by fenofibrate is partly due to different levels of circulating lipids between sexes.

The regulatory effect of fenofibrate on obesity is not mediated through leptin since PPARα-knockout mice that become obese with age are not hyperphagic [8, 10]. Instead, many reports indicate that fenofibrate-regulated increases in hepatic β-oxidation are involved in this process. FA oxidation results in a decrease in FAs available for triglyceride synthesis [78, 79]. According to Yoon et al. [20], fenofibrate elevated the transcriptional activation of PPARα target genes, acyl-CoA oxidase (ACOX), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), and thiolase in both sexes of mice [20]. However, the expression levels were much higher in males than in females, suggesting that fenofibrate exhibits sexually dimorphic activation of PPARα actions on hepatic β-oxidation, resulting in the differential energy balance with sex.

Mancini et al. [11] and Guerre-Millo et al. [10] report that fenofibrate improves obesity due to its action on FA β-oxidation in the liver and seems to act as a weight-stabilizer through its effect on liver metabolism [10, 11]. Moreover, the body weights of PPARα-deficient mice were greater than those of wild-type mice, and a marked increased amount of intra-abdominal adipose tissue was seen in PPARα-KO mice. In addition, Costet et al. [8] suggested the involvement of PPARα with a sexually dimorphic control of circulating lipids, fat storage, and obesity, in a study using male and female PPARα-null mice [8]. In contrast to these investigators, Akiyama et al. [80] provided evidence that PPARα regulates lipid metabolism but is not associated with obesity [80]. Similar to the results of Akiyama et al. [80], Yoon et al. [20] provided evidence that fenofibrate is involved in obesity, but not likely to have an effect on obesity mainly through PPARα-mediated action since it increases FA β-oxidation and decreases serum triglycerides in female mice, although their effects are much lower compared with males [20].

Overall, fenofibrate treatment affects body weight, adipose tissue mass, lipid metabolism, and hepatic β-oxidation with sexual dimorphism, but fenofibrate-regulated obesity is not directly associated with PPARα-mediated action and may be influenced by sex-related factors.

3.2. Fenofibrate Improves Male Obesity. Fenofibrate seems to suppress diet-induced obesity and severe hypertriglyceridemia caused by LDL receptor (LDLR) deficiency in male mice. The loss of LDLR increases susceptibility to diet-induced obesity and hypertriglyceridemia. Body weights and WAT mass increased in LDLR-null mice on a high fat diet compared with low fat diet controls [22, 81]. However, fenofibrate prevented the high fat diet-induced increases in body weight and WAT mass in male LDLR-null mice. The body weights of male LDLR-null mice were significantly reduced after 1 week of fenofibrate administration whereas wild-type mice showed weight decreases after 7 weeks of fenofibrate [20, 22], indicating that fenofibrate more effectively reduces body weight gain in LDLR-null mice than in wild-type mice. Interestingly, the final body weight of the fenofibrate-treated obese animals was very similar to that of lean animals on a lowfat diet. High fat diet-fed LDLR-null mice showed hepatic lipid accumulation, which was absent in the hepatocytes of mice on a low fat diet and which disappeared following fenofibrate treatment, mainly due to peroxisomal and mitochondrial β-oxidation of FAs [82, 83]. This indicates not only the prevention of body weight gain and the increased fat mobilization from WAT due to fenofibrate-induced increases of fat catabolism in the liver, but also a strong correlation between reduced body weight and decreased WAT mass by fenofibrate. In addition, fenofibrate did not affect food intake in high fat diet-induced obese LDLR-null mice. These results suggest that the increased liver activity may be paralleled by a large reduction in WAT mass, which accounts for most of the body weight reduction.

Fenofibrate also substantially decreased the increases in circulating triglycerides and total cholesterol levels, indicating that fenofibrate efficiently regulates triglyceride and cholesterol metabolism in male LDLR-null mice. Circulating triglyceride levels are thought to be regulated by the balance between its secretion and clearance. With lipoprotein catabolism suppressed, the increase in circulating triglycerides over time is indicative of the rate at which triglyceride is being secreted from the liver [84–86]. The

hepatic triglyceride secretion rate was significantly lower in fenofibrate-treated mice when Triton WR1339 was used to prevent lipolysis. These observations suggest that the reduced circulating triglyceride levels after fenofibrate treatment are due to the decreased secretion of triglycerides from the liver.

The molecular mechanisms underlying the effects of fenofibrate on obesity and lipid metabolism involve the changes in the expression of apolipoprotein C-III (apo C-III) and ACOX. LDLR-null mice fed fenofibrate showed significantly lower mRNA levels of hepatic apo C-III, an apolipoprotein that limits tissue triglyceride clearance [87, 88]. Fenofibrate-activated PPARα in the liver increased mRNA levels of ACOX, the first and rate-limiting enzyme of PPARα-mediated FA β-oxidation, which resulted in reduced triglyceride production [87].

In conclusion, fenofibrate prevents both obesity and hypertriglyceridemia through hepatic PPARα activation in male LDLR-deficient mice.

3.3. Fenofibrate Regulates Female Obesity Depending on the Presence of Ovaries. Based on the suggestion that fenofibrate inhibits body weight gain and adiposity in male LDLR-null mice, it can be hypothesized that fenofibrate improves obesity in female LDLR-null mice. Body weight gain and WAT mass were significantly increased in both female OVX and sham-operated (Sham) LDLR-null mice on a high fat diet for 8 weeks. The increases in body weight and WAT mass were higher in female OVX LDLR-null mice than in Sham mice. Interestingly, fenofibrate-treated female OVX LDLR-null mice had lower body weights and WAT mass, similar to those found in several animal models, while female Sham mice did not exhibit these fenofibrate-induced reductions [21]. In *db*/*db* mice and fatty Zucker rats, the effect of fenofibrate on body weight depends on the utilization of FA, as demonstrated by a fenofibrate-induced increase of ACOX mRNA [9]. PPARα-mediated FA β-oxidation and hydrolysis of triglycerides by fenofibrate contribute to decreased body weight and WAT mass in OVX LDLR-null mice, suggesting that fenofibrate can act as a body weight-regulator in an animal model of postmenopausal women.

Serum triglycerides and total cholesterol were significantly increased in both female OVX and Sham LDLR-null mice. However, fenofibrate treatment substantially decreased high fat diet-induced increases of triglycerides and cholesterol in both female groups [9, 87]. In parallel with serum triglyceride levels, fenofibrate upregulated hepatic ACOX mRNA levels and downregulated apo C-III mRNA levels in both OVX and Sham LDLR-null mice [87, 88]. Such changes in mRNA levels of ACOX by fenofibrate were greater in female OVX LDLR-null mice than in Sham LDLR-null mice with functioning ovaries.

However, it is not likely that the PPARα-mediated reduction in serum triglycerides directly controls obesity in female Sham LDLR-null mice, which exhibited simultaneous decreases in serum triglycerides and increases in body weight and WAT mass. Thus, the effect of fenofibrate on the body weight of female Sham LDLR-null mice cannot be explained simply in terms of an altered and enhanced flux of FAs and triglycerides, since fenofibrate increased ACOX mRNA and decreased apo C-III gene expression in this group (although this expression was lower than in the OVX group). Moreover, these changes in ACOX and apo C-III mRNA did not correlate with increased body weight and adiposity. Such conflicting data suggest the possibility that this discordance may be caused by ovarian factors.

The regulation of obesity by fenofibrate in female wild-type C57BL/6J mice is similar to that in female LDLR-null mice. Fenofibrate reduced body weight gain and WAT mass in high fat diet-fed wild-type OVX mice but failed to do so in Sham mice (Figure 5(a)) [23]. Body weights of OVX mice were found to be higher than those of Sham mice 6 weeks after commencing the high fat diet. Compared to high fat diet-fed OVX mice, fenofibrate-treated OVX mice had significantly decreased body weight gain by 6 weeks into the treatment regimen and had significantly lower body weight at 13 weeks. In addition to changes in body weight, WAT mass was significantly reduced after fenofibrate treatment, and the final WAT mass of the fenofibrate-treated OVX animals was lower than that of the OVX animals on a regular chow diet. In contrast to the OVX mice, fenofibrate did not decrease body weight gain and WAT mass increases in Sham mice. These results suggest that obesity is differentially affected by fenofibrate treatment in Sham and OVX mice.

Fenofibrate reportably acts as a weight-stabilizer through PPARα although these results were obtained using male animal models [9–11, 22]. Nevertheless, these reports suggest that fenofibrate not only prevents excessive weight gain but is also able to mobilize fat from adipose tissue by increasing fat catabolism in the liver. Notably, reductions in body weight gain and WAT mass by fenofibrate were similar in male and female OVX mice but were absent in female Sham mice.

Fenofibrate seems to differentially affect body weight and adiposity among OVX and Sham mice by a mechanism other than the modulation of leptin gene expression. Although leptin is produced only in adipose tissue and elicits satiety responses by binding to leptin receptors in the brain [89, 90], changes in leptin mRNA levels are in accordance with those in body weight and WAT mass in both female OVX and Sham mice following fenofibrate treatment. Consistent with this finding, Guerre-Millo et al. [10] reported that serum leptin concentrations positively correlated with body weight and epididymal adipose tissue mass in fenofibrate-treated male mice [10], suggesting that fenofibrate modulates body weight, not by influencing leptin gene expression and food intake, but by enhancing energy expenditure [91, 92].

Differences in PPARα target gene expression seem to explain the different effects of fenofibrate on gonad-dependent weight gain in females (Figure 5(b)). Fenofibrate not only elevated the transcriptional activation of PPARα target genes, ACOX, HD, and thiolase but also reduced apo C-III mRNA levels compared to a high fat diet alone in both groups of mice. Moreover, these alterations in expression levels were found to be more prominent in female OVX mice than in Sham mice after fenofibrate treatment. Thus, fenofibrate influences obesity via the differential activation of PPARα.

It has also been reported that ovarian steroids can affect obesity and lipid metabolism and that these effects

are likely mediated by estrogens [12]. E2 insufficiency is thought to be largely responsible for increased adiposity and circulating lipids in OVX rodents because such animals do not display obesity, adiposity, and lipid disorders when they are administered E2 replacement [17–19]. Although the administration of E2 or fenofibrate alone effectively reduces body weight gain and WAT mass in high fat diet-fed female OVX mice, fenofibrate treatment does not prevent them in female Sham mice with functioning ovaries. These results suggest the possibility that signal cross-talk may exist between PPARα and ERs in their effects on obesity and that the action of fenofibrate may be influenced by estrogens in females [25, 27, 93].

In conclusion, treatment with fenofibrate has different effects on body weight and WAT mass due in part to differentially activating hepatic β-oxidation and apo C-III gene expression between female Sham and OVX mice. These differences may provide important information about the mechanisms modulating obesity and about the actions of other lipid lowering drugs, such as fenofibrate, which are PPARα ligands in females.

3.4. The Actions of PPARα on Obesity Are Inhibited by Estrogens. My previous results show that the PPARα ligand fenofibrate reduced body weight gain and adiposity in male and female OVX mice, but not in female mice with functioning ovaries [20–23], suggesting that the actions of fenofibrate on obesity are influenced by E2.

E2 affects the ability of fenofibrate to reduce body weight gain and adiposity in female OVX mice. Mice fed a high fat diet with either fenofibrate or E2 for 13 weeks exhibited significant decreases in body weight gain and WAT mass compared to high fat diet-fed controls. These observations are supported by my previous results showing that fenofibrate stimulates hepatic FA β-oxidation in female OVX mice [21, 23], as well as by other reports showing that E2 inhibits feeding by decreasing meal size in OVX animals [94, 95]. However, these reductions were not enhanced when mice were concomitantly treated with fenofibrate and E2, indicating that E2 may inhibit the function of PPARα in female obesity [32]. Evidence from both humans and laboratory animals show that E2 plays an important role in regulating body weight and WAT mass. Ovariectomy in rodents increases WAT mass, and E2 replacement decreases WAT mass [94]. Similarly, while postmenopausal women have increased body weight gain and WAT weight, E2 decreases both of these [96, 97]. Other studies have also suggested that fenofibrate reduces body weight gain in male animal models [9–11] but does not induce decreases in body weight and WAT mass gains in female mice [20, 21, 23], suggesting that E2 may inhibit the actions of fenofibrate on body weight and WAT mass in female OVX mice.

Similarly, the combination of E2 and fenofibrate did not result in any additional beneficial effects on lipid metabolism in female OVX mice. While serum levels of total cholesterol and triglycerides were lowered in mice fed a high fat diet with either fenofibrate or E2 compared with mice fed a high fat diet alone [9, 18], the combination of E2 and fenofibrate increased levels of circulating total cholesterol and triglycerides compared with either E2 or fenofibrate alone. These results are in agreement with findings that the combination of a lipid-lowering fibrate and hormone replacement therapy (HRT) for 3 months not only had no additional benefits on the routine serum lipid or lipoprotein profiles in overweight postmenopausal women with elevated triglycerides but also increased serum triglycerides [97]. Consistent with the circulating lipid metabolism, the fenofibrate-induced decrease in hepatic lipid accumulation was also increased by E2 in female OVX mice. Mice fed a high fat diet showed considerable hepatic lipid accumulation, which was prevented by fenofibrate or E2. In contrast, mice concomitantly treated with fenofibrate and E2 showed an accumulation of triglyceride droplets. Thus, it appears that E2 inhibits fenofibrate-induced increases in fat catabolism in the liver of female OVX mice. Fenofibrate-treated OVX mice were found to have similar food intake to Sham controls whereas OVX mice given E2 showed decreased food intake. However, a combinational treatment of fenofibrate and E2 increased body weight gain, fat weight, and hepatic fat accumulation compared with fenofibrate alone, despite similar food consumption profiles between E2 and fenofibrate plus E2 groups, suggesting that E2 may affect the ability of fenofibrate to regulate energy balance.

Fenofibrate-activated PPARα has been shown to regulate the expression of a number of genes critical for FA β-oxidation and lipid catabolism. Fenofibrate upregulated ACOX, HD, and thiolase mRNA levels whereas E2 downregulated the transcriptional activation of these genes. Coadministration of fenofibrate and E2 significantly decreased ACOX, HD, and thiolase mRNA levels compared with fenofibrate treatment. These results were in accordance with serum levels of triglycerides and total cholesterol as well as body weight and WAT mass. Thus, inhibition of the actions of PPARα on body weight, WAT mass, and circulating lipid levels by E2 may be attributed, in part, to reductions in hepatic mRNA expression of PPARα-mediated peroxisomal FA β-oxidizing enzymes by E2.

Consistent with the in vivo data, E2 inhibited basal PPARα reporter gene activity as well as Wy14,643-induced reporter gene activation in NMu2Li murine liver cells transfected with PPARα, showing that E2 can modulate PPARα transactivation (Figure 6(a)). The inhibitory activity by E2 is mediated through its binding to endogenous ERs that are normally expressed in NMu2Li liver cells since it is reported that E2 does not bind directly PPARs [98]. However, the possibility that E2 directly binds to PPARα and inhibits PPARα function cannot be excluded, because no binding studies have been performed. In cells transfected with either ERα or ERβ, ERs inhibited the basal expression of PPRE-mediated reporter gene activity (Figure 6(b)). These inhibitory effects were significantly increased by E2 treatment. This is supported by results showing that PPARs can regulate ER target gene expression and that signal cross-talk between ERs and PPARs has been reported to be bidirectional [24–26, 28, 93].

Mechanistic studies revealed that the E2-ER complex was not likely to be competent for PPARα transactivation, as indicated by the inability of E2 to stimulate PPARα

recruitment of coactivators such as CBP (Figure 6(c)). Ligand-induced conformational changes that allow recruitment of coactivators, such as CBP and the dissociation of corepressors such as NCoR, are obligatory for transactivation by PPARα. Treatment of transfected CV-1 cells with Wy14,643 caused efficient CBP recruitment as evidenced by an increase in luciferase reporter gene activity. However, E2 significantly decreased Wy14,643-induced CBP association in the presence of ERα or ERβ. Thus, inhibition of PPARα transactivation by ERs was due to competition for coactivators, increased availability of corepressors, or some other mechanism. [26, 28] It has previously been shown that competition of distinct nuclear receptor for coactivator binding results in a negative cross-talk between nuclear receptors [99, 100]. These results suggest that E2 inhibition of PPARα function occurs by impairing the recruitment of transcriptional coactivators.

PPARα and ERs bind to short DNA sequences termed HREs, ERE for ERs and PPRE for PPARα [54, 101]. An ERE is an inverted repeat containing three intervening bases (AGGTCA N_3 TGACCT) whereas a PPRE is a direct repeat with one or two intervening sequences (AGGTCA $N_{1,2}$ AGGTCA). Nonetheless, these sequences contain an AGGTCA half site, which could be recognized by either ERs or PPARα. Signal cross-talk between PPAR/RXR and ERs has been reported to occur through competitive binding to ERE [24]. Therefore, the inhibition of PPARα transactivation by ERs may also have been due to their competition for PPRE.

In conclusion, in vivo and in vitro studies demonstrate that E2 inhibits the actions of PPARα on obesity through its effects on hepatic PPARα -dependent regulation of target genes and that these processes are mediated by inhibition of PPARα recruitment of coactivators by E2-activated ERs (Figure 7). PPARα ligands fibrates may act as efficient weight controllers under estrogen-free conditions. Although E2 alone decreases body weight gain and WAT mass, E2 may impair PPARα actions on obesity. Thus, these results provide a rationale for the use of fenofibrate in men and postmenopausal women with obesity and lipid disorder, but not for premenopausal women with functioning ovaries.

4. Conclusion

Obesity is the leading cause of the metabolic diseases including type 2 diabetes, atherosclerosis, and hypertension. PPARα has been the subject of intense academic and pharmaceutical research because of its ability to improve obesity-related metabolic disorders. The PPARα ligand fenofibrate seems to exhibit an antiobesity effect through FA β-oxidation in animal models although such an effect of PPARα activators has not yet been reported in humans. However, this idea is supported by several human studies showing that obese patients with impaired fat oxidation failed to lose weight, suggesting that elevated fat oxidation leads to weight loss. Interestingly, there is a sex difference in the control of obesity by fenofibrate. Fenofibrate regulates body weight and adiposity with sexual dimorphism in nutritionally induced obese male mice. Moreover, fenofibrate-induced reductions in body weight gain and WAT mass in male mice were also shown by female OVX mice, but these effects were absent in female Sham mice, suggesting the involvement of ovarian hormones in the differential regulation of obesity among these groups. In OVX mice, E2 inhibited the actions of fenofibrate-activated PPARα on obesity, due in part to reductions in hepatic expression of PPARα-mediated FA β-oxidizing enzymes by E2, a process mediated through the inhibition of PPARα coactivator recruitment by E2. These results provide a mechanism to explain why fenofibrate reduces body weight gain and adiposity in males and OVX female mice but does not regulate obesity in female mice with functioning ovaries.

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Research Article

Effects of the PPARα Agonist and Widely Used Antihyperlipidemic Drug Gemfibrozil on Hepatic Toxicity and Lipid Metabolism

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Gemfibrozil is a widely prescribed hypolipidemic agent in humans and a peroxisome proliferator and liver carcinogen in rats. Three-month feed studies of gemfibrozil were conducted by the National Toxicology Program (NTP) in male Harlan Sprague-Dawley rats, B6C3F1 mice, and Syrian hamsters, primarily to examine mechanisms of hepatocarcinogenicity. There was morphologic evidence of peroxisome proliferation in rats and mice. Increased hepatocyte proliferation was observed in rats, primarily at the earliest time point. Increases in peroxisomal enzyme activities were greatest in rats, intermediate in mice, and least in hamsters. These studies demonstrate that rats are most responsive while hamsters are least responsive. These events are causally related to hepatotoxicity and hepatocarcinogenicity of gemfibrozil in rodents via peroxisome proliferator activated receptor-α (PPARα) activation; however, there is widespread evidence that activation of PPARα in humans results in expression of genes involved in lipid metabolism, but not in hepatocellular proliferation.

1. Introduction

Gemfibrozil is a nonhalogenated derivative in the class of drugs called fibrates that include clofibrate, fenofibrate, and ciprofibrate. Since its approval by the FDA in 1982, it has been used extensively as a lipid-regulating drug and is an effective treatment of hypertriglyceridemia and hypercholesterolemia. The results of two clinical trials demonstrate that gemfibrozil has proven to be a valuable therapeutic agent in the control of coronary heart disease [1, 2]. It appears that gemfibrozil exerts hypolipidemic effects by decreasing the concentration of triglycerides [2] and low-density lipoprotein cholesterol ("bad" cholesterol) [3] and raising the concentration of high-density lipoprotein-cholesterol ("good" cholesterol) [2, 3].

In rodents, gemfibrozil and other fibrates are peroxisome proliferators, inducing a syndrome that includes enlarged livers associated with an increased number and size of hepatic peroxisomes and induction of peroxisomal and microsomal fatty acid-oxidizing enzymes including acyl CoA oxidase, carnitine acetyltransferase, and cytochrome P450 4A [4–7]. In addition to fibrates, peroxisome proliferators include selected herbicides, phthalate ester plasticizers, and endogenous long chain fatty acids [5, 8]. Peroxisome proliferators are associated with hepatocarcinogenicity in rodents. Studies with several peroxisome proliferators, including Wy-14,643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid; the prototype peroxisome proliferator), di (2-ethylhexyl) phthalate and gemfibrozil, and clofibrate have demonstrated carcinogenicity rodents [9–14].

The basis for understanding the biology of peroxisome proliferation in rodents and humans began with the discovery of the peroxisome proliferator activated receptor-α (PPARα) in 1990 [15]. Agonists for the PPARα were found to induce a battery of genes, resulting in peroxisome proliferation in the cytoplasm of rodent liver, which increased lipid catabolism via induction of peroxisomal fatty acyl-CoA β-oxidation. In humans, fibrates including gemfibrozil bind PPARα with high affinity, producing reduction in plasma

triglycerides and increased HDL concentrations [16]. These effects are thought to result from reducing apoCIII expression and induction of apolipoprotein-AI and AII expression in humans, which are under control of PPARα, and not by proliferation of peroxisomes which occurs in rodents [17]. The molecular basis of differences in response to the hepatic effects of peroxisome proliferators is hypothesized to be a combination of quantitative differences in the hepatic expression of PPARα and qualitative differences in the pattern or functionality of the downstream events that are regulated by the receptor [18, 19].

Although the biochemical and physiologic effects associated with hepatic peroxisome proliferation are thought to play a role in the hepatic toxicity and carcinogenicity in sensitive species of rodents, the mechanism of peroxisome proliferator-induced tumorigenesis and the nature of its species-selectivity are not understood [20–22]. The results of a limited number of published studies suggest that gemfibrozil is not mutagenic [12, 23]. As a result, the observed hepatocarcinogenicity is thought to be the result of indirect mechanisms. Mechanisms of PPARα-induced hepatocarcinogenicity have been recently reviewed [24]. Activation results in increase cell proliferation and decreased apoptosis. PPARα-induced oxidative stress may contribute to cell proliferation via increased signaling or may damage DNA, resulting in the initiation of carcinogenesis; the data for peroxisome proliferator-induced DNA damage are conflicting [25, 26]. Peroxisome proliferator-induced oxidative stress is thought to occur in the rodent because treatment of rodents causes large increases in the activity of the hydrogen peroxide producing peroxisomal β-oxidation enzymes while causing only minimal increases in the activity of peroxisomal catalase and decreased activity of glutathione peroxidase [27–29]. One study with Wy-14,643 revealed that hepatocarcinogenicity appears to correlate better with cell proliferation rather than peroxisome proliferation [30]. PPARα null mice have been used to evaluate the role of PPARα in rodent hepatocarcinogenicity. Wy-14,643 hepatocarinogenicity was observed in wild type mice, but not in null mice [31, 32]. In contrast, following exposure to di (2-ethylhexyl) phthalate, more liver tumors were observed in PPARnull mice compared to wild type mice [33], suggesting that PPAR-independent mechanisms may also be active in the hepatocarcinogenicity of some peroxisome proliferators. Recently, Gonzalez and colleagues have published a series of studies in wild type and humanized PPARα mice [25, 26, 32, 34, 35]. These studies demonstrate that the humanized PPARα mice are resistant to hepatocellular proliferation [25] and tumors [32] following exposure to Wy-14,643. In contrast, genes involved in peroxisomal and mitochondrial β-oxidation are induced in the wild type and humanized mice. These authors have concluded that the observed differences in the hepatocellular response are the result of differences in the disposition of let-7C microRNA (miRNA) and c-myc expression. In the wild type mice, let-7C miRNA is downregulated, resulting in the increased expression of c-myc, hepatocellular proliferation, and tumors [26, 34, 35]. In contrast, neither downregulation of let-7C miRNA nor increased expression of c-myc occurs in humanized PPARα mice, resulting in a lack of hepatocellular proliferation and tumors. These data may explain the difference in PPARα-mediated effects between rodents and humans.

The National Toxicology Program (NTP) conducted a series of 3-month feed studies in male Harlan Sprague Dawley rats, B6C3F1 mice, and Syrian hamsters to evaluate mechanisms of hepatocarcinogenicity of peroxisome proliferators; Wy-14,643 [36], gemfibrozil, dibutyl phthalate, and 2,4-dichlorophenoxyacetic acid. Gemfibrozil was included in this initiative because it interacts with the PPARα in rodents and humans as a mechanism of its pharmacological activity, and it induces hepatomegaly, peroxisome proliferation, and hepatocellular tumors in rodents. It was also of interest to evaluate whether these adverse effects were relevant to humans taking this therapeutic agent chronically. Rats and mice are commonly used in studies examining peroxisome proliferators and males are typically more sensitive than females. Hamsters were included because this species, like humans, is believed to be relatively resistant to the hepatotoxicity and carcinogenicity of peroxisome proliferators [37]. In addition to standard endpoints, the studies included assessments of hepatocyte cell proliferation, peroxisomal enzyme analysis, and analysis of lipid levels. Several investigators were awarded RO3 grants to study mechanistic aspects of peroxisome proliferator-induced hepatocarcinogenesis using tissues available from these studies [38–44]. The purpose of this manuscript is to present the effects of gemfibrozil on hepatic toxicity and lipid metabolism following exposure of rats, mice, and hamsters following subchronic exposure in feed, in the context of the NTP studies of Wy-14,643 [36].

2. Materials and Methods

2.1. Chemical and Dose Formulations. Gemfibrozil was obtained from Sigma Chemical Company (St. Louis, MO) in three lots. Lot 18F0334 was identified as gemfibrozil by infrared spectroscopy (IR) and proton nuclear magnetic resonance spectroscopy (NMR). Purity was determined to be >99% by high performance liquid chromatography (HPLC). Lot 02H0074 was found to be 98.7% pure by HPLC. Lots 18F0334 and 02H0074 were combined prior to the study and renamed as lot S040794. Purity of the combined lot was determined to be >99% by HPLC. A third lot, 104H0551, was identified by IR. Prior to the study the purity of lot S040794 and lot 104H0551 relative to a frozen reference sample of each lot was determined by HPLC to be 103.4% and 99%, respectively. Both of these lots were used in the 90-day studies. To ensure stability, the bulk chemical was stored in amber glass bottles sealed with Teflon-lined lids or sealed buckets lined with double Teflon bags, protected from light, at room temperature. During the studies, periodic reanalyses against frozen reference samples using HPLC revealed no degradation of the bulk chemical. Dose formulations were prepared by mixing gemfibrozil with feed and were stored in plastic buckets at approximately 5°C for up to 3 weeks. Homogeneity of selected dose formulations was confirmed by HPLC. Dose formulations were analyzed at the beginning, midpoint, and end of the studies. Of the dose formulations

analyzed for rats, mice, and hamsters, 96% (26/27) were within 10% of the target concentrations.

2.2. Animals and Animal Maintenance. The studies were conducted at Battelle Columbus Laboratories (Columbus, OH) in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). Male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Male B6C3F1 mice were obtained from Taconic Farms, Inc. (Germantown, NY). Male Syrian hamsters were obtained from Frederick Cancer Research and Development Center (Frederick, MD). Study animals were provided NTP-2000 open formula mean diet (Ziegler Brothers, Inc., Gardners, PA) and tap water (via automatic watering system) ad libitum. Animals were quarantined for approximately two weeks prior to the start of the studies and were approximately 8 weeks (rats and mice) or 7 weeks old (hamsters) on the first day of dosing. Study animals were distributed randomly into groups of approximate initial mean body weight and identified by tail tattoo (rats and mice) or ear tag (hamsters). Rats were housed five animals per cage. Mice and hamsters were housed individually. The animal room was maintained at a temperature of 72 ± 3°C, a relative humidity of 50 ± 15%, a light/dark cycle of 12 hours (fluorescent light) and ≥10 air changes per hour.

2.3. Study Design. Core study animals were fed diets containing 0, 10, 100, 1,000, 8,000, or 16,000 ppm (rats), 0, 10, 100, 1,000, 4,000, or 8,000 ppm (mice), or 0, 100, 1,000, 6,000, 12,000, or 24,000 ppm (hamsters) gemfibrozil for 14 weeks ($N = 10$). Additional groups of animals were designated as special study animals ($N = 15$) and were fed diets at the same concentrations for up to 13 weeks. For each species, the highest exposure concentration was based on the estimated maximum tolerated dose; in hamsters, the NTP conducted a 14-day study prior to selecting exposure concentrations for the 90-day study. Feed consumption by core study animals was recorded weekly. Core and special study animals were weighed initially, weekly, and at the end of the studies. Clinical findings were recorded weekly for core and special study animals. Other endpoints were determined as indicated below.

2.4. Clinical Chemistry. Blood for clinical chemistry was collected from special study animals on day 34 ($N = 5$) and from core study animals at the end of the studies ($N = 10$); animals were not fasted prior to blood collection. The animals were anesthetized with a mixture of carbon dioxide and oxygen, and blood was withdrawn by cardiac puncture and placed in collection tubes devoid of anticoagulant. The samples were allowed to clot and were then centrifuged; the serum was removed and stored at −70°C until analysis. The following clinical chemistry endpoints were measured in rats and hamsters: alanine aminotransferase (ALT), alkaline phosphatase (ALP), sorbitol dehydrogenase (SDH), and bile acids; mice were not evaluated for liver biomarkers due to limited serum availability. Cholesterol and triglycerides were measured in rats, mice, and hamsters.

2.5. Liver Histopathology and Weights. Following necropsy of both core and special study animals, the liver was weighed. Livers were then fixed and preserved in 10% neutral buffered formalin, trimmed and processed, embedded in paraffin, sectioned at 5-6 microns, and stained with hematoxylin and eosin for histopathological evaluation. Liver histopathology was conducted on all core study rats, mice (except 10 ppm), and hamsters. The histopathological findings were subjected to a rigorous pathology peer review including an NTP Pathology Working Group (PWG); the final diagnoses represent a consensus of peer review pathologist and the PWG. Details of these review procedures have been described by Maronpot and Boorman [45] and Boorman et al. [46].

2.6. Hepatocyte and Peroxisome Proliferation. On study days 1, 29, and 85, five special study rats, mice, and hamsters per group were implanted subcutaneously with osmotic minipumps (Model 2001, Alza Corp., Palo Alto, CA) prefilled with a 30 mg/mL solution of 5-bromo-2′-deoxyuridine (BrDU; Sigma Chemical Company, St. Louis, MO) in 0.01 N sodium hydroxide. The pumps were incubated in phosphate-buffered saline at 37°C for at least 4 hours and then implanted between 1300 and 1600 hours in animals anesthetized with 2% isoflurane via inhalation. The exact time of implantation in each animal was recorded. After 5 days (116 ± 3 hours) of BrDU exposure, the livers were evaluated for incorporation of BrDU. Approximately half of the left, right median, and anterior right lobes were fixed in 10% neutral buffered formalin for 48 hours; the remaining tissue was frozen in liquid nitrogen. The formalin-fixed liver samples, as well as a transverse section of duodenum included as an internal control, were embedded in paraffin; tissues not embedded after 48 hours of fixation were transferred to 70% ethanol. Two serial sections of each tissue were made; one slide was used for histopathologic examinations, and the second slide was stained with anti-BrDU antibody. Cell proliferation (labeled hepatocytes as a percentage of total hepatocytes) was measured by examining 2,000 hepatocyte nuclei from the left liver lobe.

A sample of the left liver lobe was collected from the BrDU animals and reserved for peroxisome proliferation analyses; approximately 1 g (rat and hamster) or 0.5 g (mouse) portions of the liver samples were prepared and analyzed for peroxisome proliferation. Peroxisome proliferation was determined in duplicate tissue extractions by measuring β-oxidation, catalase activity, and nonspecific carnitine acetyltransferase activity. Peroxisomal β-oxidation was estimated by two methods: direct measurement of acyl coenzyme A oxidase activity [47] and measurement of the β-oxidation spiral [48]. Nonspecific carnitine acetyltransferase activity was estimated by the method of Gray et al. [49, 50]. Peroxisomal catalase activity was estimated by a method derived from those of Van Lente and Pepoy [51] and Yasmineh et al. [52]. Protein concentrations were measured using the bicinchoninic method with bovine serum albumin as the standard [53]; commercially available reagents were used.

Table 1: Survival, body weights, average daily doses, and feed consumption in male core study Harlan Sprague Dawley rats, B6C3F1 mice, and Syrian hamsters following exposure to gemfibrozil in feed for 14 weeks[a].

Dose (ppm)	Survival[a]	Initial Body Weight[b] (g)	Final Body Weight[b] (g)	Body Weight Change[b] (g)	Final Body Weight (% Con)	Wk 1 Feed Consumption (g/animal/day)	Week 13 Feed Consumption (g/animal/day)	Average Daily Dose (mg/kg)
Rats								
0	10/10	236 ± 3	436 ± 5	201 ± 3	—	20.9	19.1	—
10	10/10	236 ± 2	424 ± 9	189 ± 8	97	19.9	18.6	0.6
100	10/10	235 ± 2	432 ± 8	197 ± 7	99	21.5	20.8	6
1000	10/10	237 ± 3	410 ± 16	173 ± 14*	94	20.2	20.1	60
8000	10/10	231 ± 3	350 ± 9**	119 ± 7**	80	13.0	19.3	510
16000	10/10	237 ± 2	286 ± 7**	49 ± 6**	66	7.5	22.8	1300
Mice								
0	10/10	22.1 ± 0.1	33.7 ± 0.6	11.6 ± 0.5	—	5.3	6.4	—
10	10/10	22.1 ± 0.2	35.5 ± 0.8	13.4 ± 0.6	105	6.0	5.4	1.9
100	10/10	22.1 ± 0.3	35.1 ± 0.7	13.0 ± 0.7	104	5.8	5.5	19
1000	10/10	21.2 ± 0.2	34.4 ± 0.6	12.2 ± 0.5	102	6.0	6.2	210
4000	10/10	21.5 ± 0.2	32.0 ± 0.3	10.5 ± 0.3	95	5.8	7.4	920
8000	10/10	22.0 ± 0.2	28.8 ± 0.2**	6.7 ± 0.3**	85	5.9	7.6	2100
Hamsters								
0	10/10	78 ± 1	115 ± 2	37 ± 2	—	8.8	7.3	—
100	10/10	79 ± 2	125 ± 4	46 ± 4	108	8.0	7.4	7
1000	10/10	77 ± 2	118 ± 3	40 ± 2	102	7.8	7.0	80
6000	10/10	76 ± 2	120 ± 4	44 ± 4	104	7.9	6.8	480
12000	10/10	78 ± 2	109 ± 5	30 ± 5	95	7.6	7.2	970
24000	10/10	79 ± 2	99 ± 4**	20 ± 3**	86	9.7	6.3	2000

*Significantly different ($P \leq .05$) from the control group by Williams' test; **$P \leq .01$; [a]Number of animals surviving at 3 months/number initially in group; [b]Mean ± standard error.

2.7. Statistical Methods. The Fisher exact test [54], a procedure based on the overall proportion of affected animals, was used to determine the significance of lesion incidence. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett [55] and Williams [56, 57]. Clinical chemistry and peroxisomal and hepatocyte proliferation data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley [58] (as modified by Williams, [59]) and Dunn [60]. Jonckheere's test [61] was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey [62] were examined by NTP personnel, and implausible values were eliminated from the analysis.

3. Results and Discussion

3.1. In Life Toxicity. All core study rats, mice, and hamsters survived to the end of the study. Final mean body weight gains of rats, mice, and hamsters were decreased by greater than 10% relative to controls at the highest two concentrations in rats and at the highest concentration in mice and hamsters (Table 1). Although initially reduced at 8,000 and 16,000 ppm (Table 1), feed consumption by exposed rats was similar to that by the controls by the end of the study (consumption was similar after week 2; data not shown). Feed consumption by mice and hamsters was generally similar to those by the controls; however, accurate estimates of food consumption were difficult to obtain due to extensive scattering of feed. Average daily doses that resulted from exposure to gemfibrozil are shown in Table 1. Doses ranged from 0.6–1300 mg/kg in rats, 1.9–2100 mg/kg in mice, and 7–2000 mg/kg in hamsters. No chemical-related clinical findings were observed in rats. Thinness was observed in mice (8,000 ppm) and in hamsters (2,000 and 24,000 ppm). The lack of decreased food consumption or signs of overt toxicity suggests that the decreased weight gains of exposed animals were due to alterations in lipid metabolism; similar findings were reported for Wy-14,643 [36].

3.2. Clinical Chemistry Analysis. Clinical chemistry data are presented for rats, mice, and hamsters in Table 2; mice were not evaluated for liver biomarkers due to limited serum availability.

Table 2: Clinical chemistry data for male Harlan Sprague Dawley rats, B6C3F1 mice, and Syrian hamsters following exposure to gemfibrozil in feed for 34 days (special study) or 14 weeks (core study)[a].

Rats	0 ppm	10 ppm	100 ppm	1000 ppm	8000 ppm	16000 ppm
n						
Day 34	5	5	5	5	5	5
Week 14	10	10	10	10	10	10
ALT (IU/L)						
Day 34	70 ± 5	65 ± 2	66 ± 5	67 ± 2	73 ± 4	126 ± 13*
Week 14	75 ± 6	71 ± 3	74 ± 5	101 ± 8**	219 ± 42**	178 ± 16**
SDH (IU/L)						
Day 34	21 ± 3	16 ± 2	24 ± 4	27 ± 4	26 ± 1	28 ± 7
Week 14	30 ± 4	31 ± 3	38 ± 3	54 ± 9**	141 ± 30**	86 ± 18**
ALP (IU/L)						
Day 34	706 ± 54	642 ± 13	802 ± 32	999 ± 66*	1,227 ± 66**	1,541 ± 211**
Week 14	486 ± 19	452 ± 13	560 ± 47	788 ± 53**	697 ± 30**	1112 ± 50**
Bile Salts (μmol/L)						
Day 34	29.4 ± 5.4	26.4 ± 7.2	41.4 ± 6.5	71.6 ± 8.1*	136.2 ± 11.7**	192.8 ± 27.7**
Week 14	55.3 ± 8.2	38.4 ± 2.9	54.9 ± 9.1	79.9 ± 10.4	133.4 ± 19.7**	223.4 ± 18.0**
Cholesterol (mg/dL)						
Day 34	131 ± 8	122 ± 5	150 ± 5	172 ± 5**	197 ± 11**	223 ± 14**
Week 14	120 ± 4	122 ± 4	195 ± 9**	172 ± 8**	212 ± 5**	231 ± 5**
Triglycerides (mg/dL)						
Day 34	99 ± 10	91 ± 7	91 ± 8	77 ± 7	73 ± 12	84 ± 9
Week 14	124 ± 13	121 ± 7	104 ± 13	105 ± 7	72 ± 6**	58 ± 3**
Mice	0 ppm	10 ppm	100 ppm	1000 ppm	4000 ppm	8000 ppm
Cholesterol (mg/dL)						
Day 34	159 ± 8	144 ± 5	146 ± 2	144 ± 2	180 ± 6	194 ± 3
Week 14	175 ± 3	190 ± 5	184 ± 4	212 ± 6**	219 ± 5**	216 ± 6**
Triglycerides (mg/dL)						
Day 34	142.6 ± 13.2	156.0 ± 1.47	154.8 ± 14.8	160.4 ± 5.6	122.8 ± 13.6	117.6 ± 6.6
Week 14	181.6 ± 19.8	182.1 ± 15.6	158.8 ± 10.6	134.1 ± 8.7	116.8 ± 8.8**	100.9 ± 6.0**
Hamsters	0 ppm	100 ppm	1000 ppm	6000 ppm	12000 ppm	24000 ppm
ALT (IU/L)						
Day 34	67 ± 13[b]	76 ± 14[b]	48 ± 1[b]	76 ± 7[b]	62 ± 11	58 ± 14
Week 14	73 ± 3	73 ± 10	86 ± 13	63 ± 6	71 ± 5	81 ± 10
SDH (IU/L)						
Day 34	57 ± 11[b]	59 ± 8	39 ± 2[b]	62 ± 5[b]	51 ± 9	44 ± 4
Week 14	51 ± 2	59 ± 6	73 ± 21	52 ± 4	53 ± 3	65 ± 11
ALP (IU/L)						
Day 34	265 ± 17[b]	277 ± 21[b]	241 ± 14[b]	215 ± 14[b]*	216 ± 12*	190 ± 13**
Week 14	202 ± 7	192 ± 9	177 ± 15	177 ± 7	149 ± 10**	152 ± 15**
Bile Salt (μmol/L)						
Day 34	11.3 ± 0.5[b]	10.0 ± 1.1	9.5 ± 0.9[b]	11.8 ± 1.4[b]	19.0 ± 1.4	42.4 ± 8.7**
Week 14	8.9 ± 0.7	11.3 ± 1.3[c]	11.1 ± 1.8[d]	19.5 ± 7.3	26.8 ± 5.7*	68.6 ± 10.6**
Cholesterol (mg/dL)						
Day 34	149 ± 6	143 ± 8	133 ± 5	156 ± 9	148 ± 5	152 ± 7
Week 14	149 ± 3	151 ± 8	139 ± 5	129 ± 8	153 ± 6	153 ± 7
Triglycerides (mg/dL)						
Day 34	163 ± 13	171 ± 11	240 ± 27**	263 ± 16**	192 ± 8**	258 ± 19**
Week 14	201 ± 10	210 ± 20	203 ± 17	217 ± 21	214 ± 26	375 ± 42**

* Significantly different ($P \leq .05$) from the control group by Dunn's or Shirley's test; ** $P \leq .01$; [a]Mean ± standard error, statistical tests were performed on unrounded data; [b]$n = 4$; [c]$n = 8$; [d]$n = 9$.

Table 3: Incidence and severity of liver histopathologic lesions in male core study Harlan Sprague Dawley rats, B6C3F1 mice, and Syrian hamsters following exposure to gemfibrozil in feed for 14 weeks[a].

Rats	0 ppm	10 ppm	100 ppm	1000 ppm	8000 ppm	16000 ppm
Liver, Cytoplasmic Alteration	0	7[b]**(1.0)[c]	10** (2.0)	10** (3.0)	10** (4.0)	10** (4.0)
Mice	0 ppm	10 ppm	100 ppm	1000 ppm	4000 ppm	8000 ppm
Liver, Cytoplasmic Alteration	0	NE	0	7** (1.0)	10** (2.6)	10** (3.0)
Hamsters	0 ppm	100 ppm	1000 ppm	6000 ppm	12000 ppm	24000 ppm
Liver, Glycogen Depletion	0	5[d]** (1.0)	4* (1.0)	8** (1.0)	9** (1.0)	10** (2.8)

*Significantly different ($P \leq .05$) from the control group by the Fisher exact test; **$P \leq .01$; NE = not examined; [a]$n = 10$; [b]Incidence; [c]Mean severity: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked; [d]$n = 9$.

Table 4: Relative liver weights in male special study Harlan Sprague Dawley rats, B6C3F1 mice, and Syrian hamsters following exposure to gemfibrozil in feed for 6 days, 34 days, or 13 weeks[a,b].

Rats	0 ppm	10 ppm	100 ppm	1000 ppm	8000 ppm	16000 ppm
Day 6	40.720 ± 1.154	43.574 ± 0.995	49.162 ± 1.292**	55.277 ± 1.702**	59.642 ± 1.612**	54.037 ± 0.718**
Day 34	36.76 ± 0.540	37.50 ± 0.583	45.444 ± 0.693**	53.942 ± 1.449**	68.603 ± 2.391**	75.290 ± 1.296**
Week 13	31.627 ± 1.168	33.179 ± 0.357	39.391 ± 0.866**	48.137 ± 0.523**	74.370 ± 1.212**	86.782 ± 1.678**
Mice	0 ppm	10 ppm	100 ppm	1000 ppm	4000 ppm	8000 ppm
Day 6	51.954 ± 0.788	50.291 ± 1.108	51.703 ± 2.524	54.417 ± 1.465	64.524 ± 1.391**	76.920 ± 0.441**
Day 34	41.768 ± 1.153	45.376 ± 0.687**	47.546 ± 0.742**	51.955 ± 0.327**	63.724 ± 1.036**	72.806 ± 0.749**
Week 13	41.152 ± 0.390	42.606 ± 0.862	41.689 ± 0.737	43.299 ± 1.153	60.370 ± 01.303**	71.208 ± 1.235**
Hamsters	0 ppm	100 ppm	1000 ppm	6000 ppm	12000 ppm	24000 ppm
Day 6	5.447 ± 0.286	5.119 ± 0.195	5.463 ± 0.384	5.448 ± 0.219	5.364 ± 0.157	5.214 ± 0.272
Day 34	4.238 ± 0.196	4.292 ± 0.093	4.295 ± 0.076	4.629 ± 0.195	4.723 ± 0.126*	5.341 ± 0.106**
Week 13	3.867 ± 0.086	3.718 ± 0.121	3.864 ± 0.122	3.940 ± 0.082	4.586 ± 0.067**	5.072 ± 0.157**

*Significantly different ($P \leq .05$) from the control group by Dunnett's or Williams' test; **$P \leq .01$; [a]Data are given as mg organ weight/g body weight (mean ± standard error); [b]$n = 5$.

In rats, there was a treatment-related increase (approximately 1.8-fold) in serum alanine aminotransferase activity at the highest concentration on day 34. By week 13, increases (ranging between 1.4- to 2.9-fold) in alanine aminotransferase activity occurred at the top three concentrations. Additionally, increases in sorbitol dehydrogenase activity at the highest three concentrations ranged from 1.8- to 4.7-fold. The increases in serum alanine aminotransferase and sorbitol dehydrogenase activities observed in rats would suggest a treatment-related hepatocellular effect or injury, similar to that observed for the potent peroxisome proliferators Wy-14,643 [36]. Increases in alkaline phosphatase activity and bile salt concentration, suggestive of a cholestatic event, occurred at day 34 and week 13 at the highest three concentrations. For both variables, the increases appeared to be dose-related, ranging between 1.4- to 2.3-fold for alkaline phosphatase and 2.4- to 6.7-fold for bile salts. On day 34, dose-related increases in serum cholesterol concentration occurred at the highest three concentrations; the increases were modest, ranging from 1.3- to 1.7-fold. By week 13, increases in cholesterol concentration (ranging between 1.4- to 1.9-fold) occurred in all but the lowest dose group. Conversely, at week 13, triglyceride concentration decreased by approximately 50% at the highest two concentrations.

In mice exposed for 13 weeks, a slight (20–30%) treatment-related increase in cholesterol concentration occurred at the highest three concentrations. Triglyceride concentrations, however, were decreased at the two highest concentrations; the decrease was dose-related at 35 and 44% in the 4000 and 8000 ppm dose groups, respectively.

In hamsters, increases in bile salt concentration, suggestive of a cholestatic event, occurred on day 34 and at week 13 at the highest three concentrations; the increases appeared to be dose-related, ranging between 1.7- to 7.7-fold. Alkaline phosphatase activity, another marker of cholestasis, however, was decreased at both time points at the highest three concentrations; the decreases were modest ranging between 13 to 28%. At both time points, triglyceride concentration was increased. At day 34, treatment- but not dose-related increases in serum triglyceride concentration occurred in all groups except the lowest concentration; the increases ranged from 1.2- to 1.6-fold. By week 13, triglyceride concentration was increased (1.9-fold) only at the highest concentration. There were no changes in cholesterol concentrations.

There was a clear and interesting difference between the species regarding the serum lipid (triglycerides and cholesterol) lowering effect of gemfibrozil. Rats and mice had decreases in triglycerides but increases in cholesterol concentration whereas hamsters had increases in serum triglycerides and no effect on cholesterol concentrations. The more potent peroxisome proliferator Wy-14,643 [36] had no effect on cholesterol or triglycerides in rats, caused decreases in triglycerides and increases in cholesterol (similar to gemfibrozil in both rats and mice) in mice, and caused decreases

Table 5: Hepatocyte proliferation (% BrdU labeled hepatocytes) in male special study Harlan Sprague Dawley rats, B6C3F1 mice, and Syrian hamsters following exposure to gemfibrozil in feed for 6 days, 34 days, or 13 weeks[a,b].

Rats	0 ppm	10 ppm	100 ppm	1000 ppm	8000 ppm	16000 ppm
Day 6	3.740 ± 0.154	5.303 ± 0.558**	9.333 ± 1.293**	36.244 ± 1.692**	26.061 ± 3.332**	7.152 ± 0.989[c]**
Day 34	0.783 ± 0.065	0.754 ± 0.143	0.679 ± 0.195	0.850 ± 0.199	1.300 ± 0.197	4.199 ± 0.867**
Week 13	0.451 ± 0.113	0.421 ± 0.061	0.461 ± 0.059	0.499 ± 0.076	0.608 ± 0.186	1.348 ± 0.142**
Mice	0 ppm	10 ppm	100 ppm	1000 ppm	4000 ppm	8000 ppm
Day 6	2.366 ± 0.345	1.414 ± 0.546	1.141 ± 0.239	1.766 ± 0.768	2.340 ± 0.842	4.449 ± 0.555
Day 34	0.896 ± 0.240	1.488 ± 0.240	1.599 ± 0.201	1.768 ± 0.385	2.097 ± 0.350	1.627 ± 0.388
Week 13	0.705 ± 0.069	1.167 ± 0.274	1.052 ± 0.157	1.240 ± 0.315	1.062 ± 0.122	0.958 ± 0.130
Hamsters	0 ppm	100 ppm	1000 ppm	6000 ppm	12000 ppm	24000 ppm
Day 6	2.042 ± 0.863	1.123 ± 0.117	4.323 ± 1.652	6.108 ± 3.669	2.572 ± 0.990	3.594 ± 1.598
Day 34	2.713 ± 0.582	2.148 ± 0.665	1.120 ± 0.249	3.932 ± 0.267	3.467 ± 0.962	1.566 ± 0.473
Week 13	1.176 ± 0.267	3.962 ± 0.848*	4.787 ± 1.085*	1.969 ± 0.169	3.625 ± 1.637	2.450 ± 0.478

**Significantly different ($P \leq .01$) from the control group by Shirley's test; [a]Mean ±standard error; [b]$n = 5$; [c]$n = 4$; BrdU: bromodeoxyuridine.

in serum cholesterol and triglycerides in hamsters. Hamsters are a better model for human lipoprotein metabolism that rats or mice, as hamsters, like humans, make cholesterol ester transfer protein (CETP) [16, 63]. In addition, hamsters have a similar hepatic sterol synthesis rate to humans; the rate is much higher in rats and mice [64]. It is unclear why lipid-lowering effects were not observed in hamsters following exposure to gemfibrozil in the present study.

3.3. Liver Histopathology and Weights. The incidence of hepatocyte cytoplasmic alteration was significantly increased in all exposed groups of rats and in mice exposed to 1000 ppm or greater (Table 3). The severity of this lesion was increased in rats exposed to 100 ppm or greater and in mice exposed to 4000 or 8000 ppm. A dose-related increase in severity was observed in both rats and mice. Hepatocyte cytoplasmic alteration was characterized by prominently increased cytoplasmic granularity and eosinophilia with some evidence of hepatocyte enlargement in severe cases. This change was generally diffuse but in some cases, the distribution was centrilobular to midlobular and of minimal severity. The granularity observed in the hepatocytes was considered consistent with the known hepatocellular appearance of peroxisome proliferation in the liver. Hepatocyte cytoplasmic alteration was not observed in hamsters, indicating a lack of morphological evidence of peroxisome proliferation; however, hepatic glycogen depletion was significantly increased in all exposed groups and increased in severity at the highest concentration (Table 3). Glycogen depletion was characterized by a decrease or absence of clear vacuoles in the cytoplasm of hepatocytes. The glycogen content of the liver is variable and may fluctuate depending on the physiological state of rodents. While glycogen depletion is commonly seen in animals that have been fasted, it may also be observed due to the pharmacologic or toxic effects of xenobiotic exposure.

Absolute and relative liver weights were recorded in core (data not shown) and special study animals. Table 4 presents the relative liver weight data for special study animals on day 6, day 34, and week 13. In all three species, the maximum increase in relative liver weight was observed at week 13. At all time points, the relative liver weights of rats exposed to 100 ppm or greater were significantly increased. On day 6, the largest increase was observed at 8000 ppm, while the increases at 1000 ppm and 16000 ppm were similar. In mice, relative liver weights were increased at all durations at the highest two exposure concentrations and at all concentrations on day 34. In hamsters, more modest, but significant increases were observed at the highest two concentrations on day 34 and week 13. The largest increases in relative liver weight were observed in rats (up to 2.8-fold) and the smallest increases were in hamsters (up to 1.3-fold); liver weights in mice were increased at up to 1.7-fold.

3.4. Hepatocyte and Peroxisome Proliferation. There were significant increases in hepatocyte cell proliferation, measured as BrdU labeling of hepatocytes, in rats at all exposure durations (Table 5). Cell proliferation was increased in all exposed groups of rats on day 6. The greatest increases were observed in the 1000 ppm (9.7 fold) and 8000 ppm (7.0 fold) groups, while the increases at 100 ppm (2.5 fold) and 16000 ppm (1.9 fold) were similar. This pattern, which is similar to that observed for relative liver weight on day 6, was not observed at the day 34 and week 13 exposure durations, as the greatest increases were at the highest concentration at these durations. The magnitude of the maximum increase in cell proliferation was less with increasing exposure duration (9.7-fold on day 6, 5.4-fold on day 34, and 3.0-fold at week 13). There were no biologically significant increases in hepatocyte proliferation in mice or hamsters. The lack of an increase in mice was noteworthy, given that increases in other endpoints were observed in both rats and mice. In the NTP studies of Wy-14,643, increased hepatocyte proliferation was observed in all three species at all three exposure durations, with greater increases in rats and mice relative to hamsters [36]. In rats and hamsters, the magnitude of the response was lower at longer durations; however, the response was sustained in mice. In a feed study evaluating hepatocyte proliferation with Wy-14,643 and DEHP, at exposure durations out to one year, a sustained proliferative response was observed with Wy-14,643, but not DEHP [30];

Table 6: Hepatic peroxisomal enzyme activities in male special study Harlan Sprague Dawley rats, B6C3F1 mice, and Syrian hamsters following exposure to gemfibrozil in feed for 6 days, 34 days, or 13 weeks[a,b].

Rats	0 ppm	10 ppm	100 ppm	1000 ppm	8000 ppm	16000 ppm
			Acyl CoA oxidase (nmol DCF/minute per mg)			
Day 6	1.5 ± 0.2	1.8 ± 0.7	1.3 ± 0.2	2.0 ± 0.1	6.9 ± 0.7**	11.1 ± 1.0**
Day 34	1.8 ± 0.2	1.9 ± 0.1	2.5 ± 0.5	5.2 ± 0.4**	16.5 ± 2.6**	22.1 ± 2.4**
Week 13	1.6 ± 0.1	1.6 ± 0.1	2.9 ± 0.4*	6.8 ± 0.5**	27.2 ± 0.9**	33.2 ± 1.2**
			β-Oxidation (Lazarow method) (nmol NADH/minute per mg)			
Day 6	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	2.3 ± 0.3	12.2 ± 1.0**	19.8 ± 2.0**
Day 34	1.2 ± 0.2	1.0 ± 0.1	2.3 ± 0.3*	10.0 ± 1.3**	37.9 ± 6.0**	55.8 ± 7.0**
Week 13	1.4 ± 0.2	1.4 ± 0.1	2.4 ± 0.3*	16.9 ± 1.3**	75.2 ± 4.8**	89.9 ± 4.2**
			Carnitine acetyltransferase (nmol reduced CoA/minute per mg)			
Day 6	0.8 ± 0.1	0.9 ± 0.1	1.5 ± 0.5**	2.0 ± 0.1**	11.3 ± 1.2**	15.9 ± 1.7**
Day 34	0.7 ± 0.1	0.7 ± 0.1[c]	2.3 ± 0.2**	7.0 ± 1.3**	18.6 ± 4.0**	21.8 ± 3.1**
Week 13	0.6 ± 0.1	0.8 ± 0.1	3.3 ± 0.9**	13.9 ± 2.3**	55.6 ± 5.2**	42.8 ± 5.1**
			Catalase (nmol NADPH/minute per mg)			
Day 6	253 ± 17	234 ± 17	177 ± 13	162 ± 9	302 ± 11	292 ± 18
Day 34	289 ± 23	253 ± 16	222 ± 18	329 ± 29	438 ± 30*	476 ± 26*
Week 13	274 ± 28	281 ± 6.0	253 ± 22	378 ± 28*	523 ± 24**	508 ± 29**
Mice	0 ppm	10 ppm	100 ppm	1000 ppm	4000 ppm	8000 ppm
			Acyl CoA oxidase (nmol DCF/minute per mg)			
Day 6	1.2 ± 0.1	1.0 ± 0.0	1.1 ± 0.1	2.5 ± 0.1*	8.2 ± 1.8**	16.5 ± 2.2**
Day 34	1.2 ± 0.1	1.4 ± 0.2	1.0 ± 0.3	2.7 ± 0.5*	8.5 ± 0.7**	15.5 ± 0.4**
Week 13	1.2 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	1.7 ± 0.1*	10.9 ± 1.3**	18.0 ± 1.7**
			β-Oxidation (Lazarow method) (nmol NADH/minute per mg)			
Day 6	0.5 ± 0.2[c]	1.0 ± 0.2	0.8 ± 0.2	1.9 ± 0.1[c]**	15.3 ± 0.5[c]**	30.6 ± 1.3**
Day 34	1.0 ± 0.3	1.0 ± 0.2	0.9 ± 0.1[c]	1.9 ± 0.1*	19.8 ± 1.1**	45.4 ± 1.3**
Week 13	1.2 ± 0.1[c]	1.2 ± 0.1	1.2 ± 0.2	1.9 ± 0.2	21.3 ± 0.6**	42.2 ± 1.5**
			Carnitine acetyltransferase (nmol reduced CoA/minute per mg)			
Day 6	1.2 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	2.6 ± 0.3**	11.1 ± 2.0**	17.2 ± 0.5**
Day 34	1.7 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	2.7 ± 0.3	15.7 ± 1.3*	24.6 ± 1.4**
Week 13	1.5 ± 0.2	1.7 ± 0.1	1.8 ± 0.3	2.8 ± 0.3**	18.1 ± 0.9**	24.8 ± 1.0**
			Catalase (nmol NADPH/minute per mg)			
Day 6	98.3 ± 6.2	96.8 ± 4.2	95.8 ± 2.8	96.0 ± 2.6	187.8 ± 27.4	314.7 ± 8.7**
Day 34	98.3 ± 4.0	88.1 ± 3.9	86.6 ± 3.8	89.3 ± 6.3	225.9 ± 8.8	289.2 ± 4.3**
Week 13	74.8 ± 4.4	75.4 ± 7.1	84.2 ± 10.5	78.7 ± 7.4	238.8 ± 8.4**	302.1 ± 11.3**
Hamsters	0 ppm	100 ppm	1000 ppm	6000 ppm	12000 ppm	24000 ppm
			Acyl CoA oxidase (nmol DCF/minute per mg)			
Day 6	2.4 ± 0.2	2.1 ± 0.1	2.5 ± 0.2	3.1 ± 0.4	4.0 ± 0.2*	4.4 ± 0.6*
Day 34	2.1 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	3.1 ± 0.2*	3.2 ± 0.3*	4.6 ± 0.4**
Week 13	2.2 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	2.9 ± 0.1**	3.2 ± 0.2[c]**	3.7 ± 0.4**
			β-Oxidation (Lazarow method) (nmol NADH/minute per mg)			
Day 6	1.9 ± 0.1	1.9 ± 0.5	1.7 ± 0.1	2.2 ± 0.4[d]	2.7 ± 0.2[c]	3.0 ± 0.2**
Day 34	2.2 ± 0.1	1.8 ± 0.3	2.0 ± 0.3	2.0 ± 0.2	1.6 ± 0.3[c]	2.0[e]
Week 13	2.1 ± 0.1	2.0 ± 0.1	1.6 ± 0.3	1.6 ± 0.2	2.0 ± 0.2[c]	2.2 ± 0.5[f]
			Carnitine acetyltransferase (nmol reduced CoA/minute per mg)			
Day 6	8.0 ± 0.5	8.1 ± 0.2	7.6 ± 0.6	10.8 ± 0.4**	12.8 ± 0.9**	14.1 ± 1.6**
Day 34	7.0 ± 0.2	6.1 ± 0.2	8.2 ± 0.5	7.4 ± 0.4	9.3 ± 0.4*	17.2 ± 2.1**
Week 13	6.8 ± 0.4	6.0 ± 0.2	7.5 ± 0.8	8.2 ± 0.3	10.8 ± 2.0*	14.1 ± 2.2**

Table 6: Continued.

Rats	0 ppm	10 ppm	100 ppm	1000 ppm	8000 ppm	16000 ppm
	Catalase (nmol NADPH/minute per mg)					
Day 6	273 ± 21	304 ± 9	249 ± 25	260 ± 37[c]	251 ± 17	226 ± 11
Day 34	302 ± 24	312 ± 22	285 ± 12	284 ± 22	258 ± 16	261 ± 34
Week 13	291 ± 19	272 ± 17	269 ± 26	270 ± 10	246 ± 14	252 ± 13[c]

**Significantly different ($P \leq .01$) from the control group by Shirley's test; [a]Mean ± standard error; [b]$n = 5$; [c]$n = 4$; [d]$n = 3$; [e]$n = 1$, no standard error presented because only one sample available; [f]$n = 2$.

this sustained proliferation with Wy-14,643 is likely reflective of its potency. The lack of a sustained proliferative response in rats with gemfibrozil in the present study is similar to that observed with DEHP and other peroxisome proliferators, indicating less potency relative to Wy-14,643.

Peroxisomal enzyme activities are shown in Table 6. In rats, mice, and hamsters, Acyl CoA Oxidase, β-oxidation, and carnitine acetyl transferase were generally increased with increasing concentration; however, the effect was not as pronounced in hamsters. In rats and mice, the greatest increases relative to controls were generally observed at week 13. In rats, these enzymes were increased at 100 ppm or greater by week 13. In mice, these enzymes were generally increased at the highest three concentrations. In hamsters, Acyl CoA oxidase and carnitine acetyltransferase were increased at the highest two concentrations at all durations and at the highest three concentrations on day 34 and week 13 (Acyl CoA oxidase) or day 6 (carnitine acetyltransferase). β-oxidation was increased only at the top two concentrations on day 6. Maximum increases in Acyl CoA oxidase and carnitine acetyltransferase were similar between rats and mice, while the increase in β-oxidation in rats was much greater than in mice. In general, increases in catalase were observed only at higher concentrations and longer durations relative to the other enzymes in rats and mice; the lower induction of catalase relative to hydrogen peroxide generating enzymes is consistent with previous reports. Increases in catalase were not observed in hamsters. The greater observed increases in hydrogen peroxide generating enzymes relative to increases in catalase, which removes hydrogen peroxide, are consistent with previous studies on peroxisome proliferators, supporting the hypothesis that hepatocarcinogenesis may arise due to a net increase in hydrogen peroxide and subsequent oxidative stress. A generally similar pattern of increased enzyme activities was observed with Wy-14,643 [36] except that responses occurred at lower concentrations.

3.5. Comparison of Results with Gemfibrozil Cancer Bioassay. In a previous cancer bioassay of gemfibrozil [12], male and female albino CD rats and CD-1 mice were exposed to 0, 30, or 300 mg/kg for 104 weeks (rats) or 78 weeks (mice). The authors stated that gemfibrozil was a liver carcinogen in male rats, but not in female rats or in mice of either sex. In rats, there was a clear and significant increase in benign liver neoplastic nodules at 300 mg/kg and an increased number of liver carcinomas at both 30 and 300 mg/kg. In mice, there was a significant increase in hepatocellular carcinomas at 30 mg/kg, but not 300 mg/kg. In the present study, a dose of 300 mg/kg would result from exposure to between 1000 (60 mg/kg) and 8000 (510 mg/kg) ppm in rats and 1000 (210 mg/kg) and 4000 (920 mg/kg) ppm in mice. At these concentrations, significant increases in hepatocyte cytoplasmic alteration, relative liver weights, hepatocyte proliferation (rats only), and hydrogen peroxide producing peroxisomal enzyme activities were increased. In general, despite the fact that the dose range was higher in mice than in rats, there were greater increases in these endpoints in rats; this is especially the case with hepatocyte proliferation, which was not increased in exposed mice. Thus, it appears that the species susceptibility to liver tumors correlates with that of peroxisome-proliferation-related hepatic effects. However, the observed differences in response may be the result of different exposure durations.

3.6. Studies by Investigators Utilizing NTP Tissues. Several investigators utilized tissues from the NTP peroxisome project studies to evaluate mechanisms of peroxisome proliferator-induced hepatocarcinogenicity with selected compounds. These studies typically evaluated oxidative stress-related mechanisms of action. Differences in species susceptibility between rats and hamsters were observed with several endpoints, including selenium dependent glutathione peroxidase activity, which was increased in hamsters and decreased in rats following exposure to Wy-14,643, GEM, and DBP [38]; activation of NFkappaB, occurred in rats primarily with Wy-14,643 but to a lesser extent with Gem and DBP, but not in hamsters [39]; and polymerase-β, Ref-1, and PNCA were increased in rats but either observed at trace levels (polymerase-β) or decreased (Ref-1 and PCNA) in hamsters, following exposure to Wy-14,643 [42]. In contrast, some endpoints did not reflect species differences, including glutathione-S-transferase and glutathione reductase activities following exposure to Wy-14,643 and DBP [38], activation of several redox-sensitive transcription factors, including AP-1 early growth response gene 1 and heat shock factors 1 and 2 following exposure to Wy-14,643, GEM or DBP [40], expression of the proapoptotic protein Bax following exposure to Wy-14,643, GEM, and DBP [42], and antioxidant capacities with dibutyl phthalate, gemfibrozil, or Wy-14,643 [41]. Exposure of rats and mice to Wy-14,643 increased the expression of several base excision repair enzymes, but not the expression of enzymes that are not involved in the repair of oxidative DNA damage [44]. The other compounds induced weaker or no increases in the expression of these enzymes. In another study, WY, Gem, DBP, and 2,4-D were evaluated for their ability to alter the

methylation and expression of the c-myc protooncogene in mice [43]. All four peroxisome proliferators caused hypomethylation of the c-myc gene in the liver, while only Wy-14,643 increased the level of c-myc protein. Collectively, these studies provide some insight regarding oxidative stress-related mechanisms of peroxisome proliferators and species differences in susceptibility.

3.7. Comparison of PPARα-Mediated Effects in Rodents and Humans. Recent studies by Gonzalez and colleagues using humanized PPARα mice have provided some information on the mechanism of PPARα-hepatocarcinogenicity in rodents and species differences between rodents and humans following exposure to rodent peroxisome proliferators [25, 26, 32, 34, 35]. Hepatocellular proliferation and neoplasms were observed in wild type, but not humanized PPARα mice. A proposed mechanism of the hepatic proliferative effects involves downregulation of let-7C miRNA, resulting in the increased expression of c-myc, which in turn results in increased hepatocellular proliferation and tumors [26, 34, 35]. In contrast, these biochemical and morphologic effects are not observed in humanized PPARα mice. In mice with both receptor types, induction of genes involved in peroxisomal and mitochondrial β-oxidation were observed. These data may explain the difference in PPARα-mediated effects between rodents and humans.

Several studies evaluated the pleiotropic responses to prolonged (from 14 days up to 13 weeks) oral administration of relatively high doses of peroxisome proliferators (500 to 2500 mg/kg) in several species of nonhuman primates [65–67]. In contrast to results found in rodents, no significant increases in liver weight, induction of peroxisomal enzymes, or proliferation of peroxisomes were reported. Studies that examined patients treated with relatively more potent PPs (e.g., clofibrate, gemfibrozil, or fenofibrate), for prolonged periods of time (i.e., years) are more consistent with the idea that humans do not exhibit peroxisome proliferation in response to exposure to PPs. Similar to findings made in hemodialysis patients, a marginal 50% increase in liver peroxisome number, but not in peroxisome volume, is reported in humans treated with clofibrate [68]. In contrast, the majority of studies examining the effect of PP administration in humans have consistently shown no change in hepatic peroxisome proliferation in liver (reviewed in [24]).

There are no known reports of long-term carcinogenesis studies with PPs in nonhuman primates. Several large epidemiological studies that examined the relationship between chronic treatment with lipid-lowering PPs gemfibrozil and clofibrate did not find an association with liver cancer (reviewed in [24]). Collectively, human epidemiological studies have not shown an association between liver cancer and treatment with PPs [69].

The molecular mechanism by which hypolipidemic fibrates and antidiabetic thiazolidinediones exert their therapeutic effect in humans is similar to the way peroxisome proliferators exert their toxicity in rodents, namely, by activation of the PPAR family of receptors. In response to exposure to a PP chemical, the mRNA and protein levels of numerous enzymes are increased in rodents, including the enzymes in the peroxisome per se but also microsomal cytochrome CYP4A. Primary organs involved in this pleiotropic response are liver, kidney, and heart. A receptor responsible for activating these diverse effects was identified, termed the peroxisome proliferator-activated receptor (PPAR) and was demonstrated to belong to the nuclear receptor superfamily that includes the estrogen, progesterone, and retinoic acid receptors. Members of the PPAR family of receptors include PPARα, PPARβ/δ, and PPARγ, which have different tissue distributions, abundances and functions in lipid metabolism during different stages of development. PPARγ mRNA has been detected in greatest amounts in human heart, placenta, lung, and kidney, but has also been identified in human prostate, testis, and ovary [70, 71].

PPARα mediates gene activation through binding to a DNA response element (PPRE) (a DR-1 response element) upstream from all genes that are known to respond to PPs. These include genes in the peroxisome mentioned above as well as cytochromes CYP4A and fatty acid binding protein. The other members of the PPAR superfamily (PPARβ/δ and γ) bind to and activate similar PPRE but in different tissues. PPAR-ligand complex binds to the PPRE upstream of the LPL and Apo A I and -II genes in humans, whereas it binds upstream and activates different genes in rodents, namely, those genes responsible for the peroxisome proliferation response. The increased lipoprotein lipase and apolipoprotein (apo) A-I and apoA-II induction increase plasma HDL and increase triglyceride mobilization. In rats PPARα activation decreases apoA-I and apoA-II gene expression and lowers plasma HDL [72]. In humans, HDL cholesterol is elevated after fibrate treatment due to increased lipolysis of triglyceride-rich lipoproteins and redistribution of lipid components to HDL.

Although the PPRE is almost identical in rodents (TGCCCTTCCCCC) and humans (TGCCCTTCCCCC), the location in the genome of the PPRE is different across species resulting in vastly different genes expressed following activation of the PPAR family.

The human receptor appears to be activated by certain fatty acids and eicosanoids and thiazolidinedione antidiabetic drugs, although it appears to be only weakly activated by classical PPs such as, Wyeth-14,643 nafenopin and clofibric acid [71]. Endogenous ligands for PPARs include most straight-chain fatty acids, substituted fatty acids, and the acyl-CoA esters of fatty acids, and arachidonic acid derived prostaglandins and eicosanoids [73].

In humans, like rodents, fibrate drugs used in the treatment of hyperlipidemia are thought to activate PPARα in the liver. However, unlike rodents, activation of PPARa in humans does not result in peroxisome proliferation but results in increased apolipoprotein A-II and lipoprotein lipase transcription, and reduced apolipoprotein C-III, which is key to their mechanism of action to lower serum triglycerides [74–76] as well as induction of fatty acid transport protein and acyl-CoA synthetase [77]. (Apo C-III is a major component of very low-density lipoproteins (VLDL) and inhibits lipoprotein lipase and inhibits clearance of lipoproteins by the liver).

The antidiabetic agents in the thiazolidinediones activate human PPARγ in adipose tissue where lipoprotein lipase expression is also increased. LPL is transcriptionally activated and results in increased lipolytic activity and a decrease in serum triglycerides in humans without an increase in peroxisome activity seen in rodents, again due to the location of the PPARγ response element upstream of the LPL gene [75].

4. Conclusions

The present NTP studies confirm the induction of hepatomegaly and hepatocyte and peroxisome proliferation and alteration of lipids following exposure to gemfibrozil. Similar to NTP studies with Wy-14,643 [36], these studies also present data on hamsters, which were considered, like humans, to be nonresponsive to PPARα-mediated effects on hepatic and peroxisome proliferation, similar to primates and humans. Based on these data, it is apparent that rats are most responsive to the hepatic effects of gemfibrozil, while mice are intermediate and hamsters are the least responsive; however, the increases in peroxisomal enzymes indicate peroxisome proliferation is induced in hamsters. In all three species, the pattern of peroxisomal enzyme is consistent with previous reports, with greater increases in hydrogen peroxide-generating enzymes compared to catalase. The greater sensitivity to the induction of hepatic peroxisome and hepatocellular proliferation in rats compared to mice may explain the differences in liver carcinogenicity between the two species observed in a previous study. Gemfibrozil produced alterations of lipid metabolism in each species; the effects and rats and mice were similar and distinct from hamsters.

It is clear from several investigators that humans possess a functional PPAR family of receptors. It is also clear that they regulate different genes relative to the receptor family in rodents, and that the human PPAR receptor is activated by xenobiotic drugs and chemicals. What is less clear is the relative potency of phthalates to activate the hPPAR family compared to therapeutic agents as well as compared to endogenous activators, and what such activation, if any, would result that may have deleterious effects in humans [11]. Indeed, in two recent reviews of the medical significance of PPARs, it was reported that since PPAR does not induce peroxisomes in humans the term peroxisome proliferator per se in a medical context is a misnomer ([21, 78] and references contained therein). An excellent review of the mechanism of action of fibrates in humans was published recently [79]. Recently published studies using humanized PPARα mice have provided mechanistic insights into the observed hepatocarcinogenicity in rodents and on differences between rodents and humans [25, 26, 32, 34, 35].

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Research Article

Developmental Effects of Perfluorononanoic Acid in the Mouse Are Dependent on Peroxisome Proliferator-Activated Receptor-Alpha

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Perfluorononanoic acid (PFNA) is one of the perfluoroalkyl acids found in the environment and in tissues of humans and wildlife. Prenatal exposure to PFNA negatively impacts survival and development of mice and activates the mouse and human peroxisome proliferator-activated receptor-alpha (PPARα). In the current study, we used PPARα knockout (KO) and 129S1/SvlmJ wild-type (WT) mice to investigate the role of PPARα in mediating PFNA-induced *in vivo* effects. Pregnant KO and WT mice were dosed orally with water (vehicle control: 10 ml/kg), 0.83, 1.1, 1.5, or 2 mg/kg PFNA on gestational days (GDs) 1–18 (day of sperm plug = GD 0). Maternal weight gain, implantation, litter size, and pup weight at birth were unaffected in either strain. PFNA exposure reduced the number of live pups at birth and survival of offspring to weaning in the 1.1 and 2 mg/kg groups in WT. Eye opening was delayed (mean delay 2.1 days) and pup weight at weaning was reduced in WT pups at 2 mg/kg. These developmental endpoints were not affected in the KO. Relative liver weight was increased in a dose-dependent manner in dams and pups of the WT strain at all dose levels but only slightly increased in the highest dose group in the KO strain. In summary, PFNA altered liver weight of dams and pups, pup survival, body weight, and development in the WT, while only inducing a slight increase in relative liver weight of dams and pups at 2 mg/kg in KO mice. These results suggest that PPARα is an essential mediator of PFNA-induced developmental toxicity in the mouse.

1. Introduction

Perfluorinated alkyl acids (PFAAs) are a family of chemicals that have a fatty acid-like carbon backbone saturated with fluorine and a functional group at the end. They are surfactants used in many consumer and industrial applications such as waterproofing and stain repellent on clothing, carpets, and other fabrics, oil repellent on food packaging, fire-fighting foams, paints, adhesives, hydraulic fluids, among others [1–4]. Their widespread use in consumer and industrial products is matched by their global presence in the environment [2, 5, 6] and in wildlife and humans [7–12]. The ubiquitous presence of these chemicals, especially in human sera, has led to concern about their safety. The two most common PFAAs, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), have been found in laboratory animals to induce hepatotoxicity, carcinogenicity, immunotoxicity, disruption of thyroid hormone levels, and developmental effects including prenatal and neonatal mortality, stunted mammary gland development, developmental delay, and reduced body weight (reviewed [6, 13]). Although the manufacture of PFOS was phased out in the United States and the manufacture of PFOA is being phased out, alternative PFAAs have been marketed for use.

Perfluorononanoic acid (PFNA) is a 9-carbon member of the PFAA family found in the environment and in serum at levels much lower than those of PFOA or PFOS. Nevertheless, levels of PFNA in human serum have risen in the last several years and currently stand at around 1 ng/ml [7, 14]. Its presence in human serum has been shown to correlate

with PFNA ingested from food and water [15, 16]. Few studies have investigated its toxicity. In vitro studies found PFNA to be cytotoxic in HCT-116 cells [17], and hepatotoxic [18]. PFNA was also found to be immunotoxic *in vivo* [19, 20]. More recently, PFNA was found to induce developmental toxicity in mice when administered throughout the gestational period [21]. Adverse effects of exposure to PFNA during gestation include reduced postnatal survival at 5 mg/kg/day, delayed eye opening, delayed puberty, increased liver weight, and reduced body weight at 3 and 5 mg/kg/day.

One of the mechanisms implicated in the toxicity of the PFAAs is the activation of peroxisome proliferator-activated receptor-alpha (PPARα). PPARα is a nuclear receptor that plays a role in regulating lipid and glucose homeostasis, cell proliferation and differentiation, and inflammation [22]. PPARα activation is thought to be responsible for PFOA-induced hepatotoxicity in rodents [23] and certain immunotoxic effects [20, 24, 25]. In addition to PFOA, a number of other PFAAs activate PPARα *in vitro* [26–28]. PPARα may mediate developmental processes, since PPARα is present during murine development [29]. The developmental toxicity of PFOA in mice, including neonatal lethality, delayed eye opening, and reduced body weight, was found to be dependent on PPARα [30] although developmental toxicity of PFOS was not [31]. PPARα may also mediate PFNA effects. Evidence of PPARα activation was found in livers of mice exposed to PFNA during fetal development [21]. PFNA also activates PPARα *in vitro* and was the most effective of the PFAAs tested in activating both human and murine PPARα in transfected COS-1 cells [28]. It is therefore logical to postulate that the developmental toxicity of PFNA, like PFOA, may also be dependent on PPARα.

In the current study, we sought to determine whether PFNA-induced developmental toxicity in the mouse requires expression of PPARα. Pregnant 129S1/SvlmJ wild-type (WT) and PPARα knockout (KO) mice were given PFNA during gestation, and indices of fertility and neonatal development, along with serum levels of PFNA, were evaluated. We report that the developmental effects of PFNA including pup survival, eye opening, and body weight are dependent on PPARα and that hepatomegaly is primarily PPARα dependent but may utilize other pathways as well.

2. Materials and Methods

2.1. Animals. Male and female wild-type (WT) 129S1/SvlmJ mice (stock no. 002448) and PPARα knockout (KO) mice on a 129S1/SvlmJ background (Ppara-tm1Gonz/J, stock no. 003580) were obtained from Jackson Laboratories (Bar Harbor, ME). WT and KO mice were kept in breeding colonies in the EPA Reproductive Toxicology Facility, Durham, NC. Colony animals were group housed by sex in Tecniplast cages (Tecniplast USA, Exton, PA) with Beta-chip hardwood bedding (Northeastern Products, Warrensburg, NY) in a closed ventilation system, provided pelleted mouse chow (LabDiet 5001, PMI Nutrition International LCC, Brentwood, MO) and tap water *ad libitum*, and kept in an atmosphere of 68–74°F and 40–60% humidity with a 12-hour light-dark cycle. All animal studies were conducted in accordance with guidelines established by the USe EPA ORD/NHEERL Institutional Animal Care and Use Committee. Procedures and facilities were consistent with the recommendations of the 1996 NRC "Guide for the Care and Use of Laboratory Animals", the Animal Welfare Act, and Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

2.2. Study Design and Protocol. The study was conducted in four blocks with WT and KO represented in each block. WT and KO females were mated overnight to males of their respective strain, one mating pair per cage. Females were checked for vaginal plugs the following morning and plug positive animals were weighed, randomly assigned to treatment groups, and housed individually in regular ventilated polypropylene cages. Day of plug was considered gestational day (GDs) 0. Animals of each strain were weighed and dosed by oral gavage once daily on GD 1–18 with either water (vehicle control: 10 ml/kg) or PFNA (CAS# 375-95-1; 97% pure; Aldrich, St. Louis, MO) at 0.83, 1.1, 1.5, or 2.0 mg/kg, based on previous studies with PFNA and PFOA [21, 30]. Dosing solutions were prepared by dilution, fresh daily immediately before dosing. At term, adult females were checked twice daily for the presence of pups. Adult females with pups or those were pregnant were called dams. Day of birth was considered postnatal day (PND) 0. Dams and pups were monitored on a daily basis. The numbers of live and dead pups were recorded twice daily, and live pups were weighed by sex on postnatal days 0, 1, 2, 3, 7, 10, 14, and 21 (weaning). Pups were monitored for eye opening daily from PND 11 until all eyes were open. Eye opening is described as the percentage of pups per litter having both eyes completely open and was identified by technicians trained by demonstration and protocol to eliminate subjectivity. All animals on study were sacrificed for necropsy on PND 21 (42 days postcoitus for nonpregnant adult females). Body and liver weights were measured from each adult female and from 2 pups per litter. Blood was collected from each dam individually and from all pups pooled by litter. Serum was extracted and stored at −20°C. Uteri were collected from all adult females, stained with 2% ammonium sulfide, and uterine implantation sites were counted [32].

2.3. Serum Analysis of PFNA. Analysis of PFNA in serum was performed using a modification of a method previously described in [33]. For the current study, 25 μl of serum was placed in a 6 ml polypropylene tube, deproteinized with 1 ml of 0.1 M formic acid, and vortexed. Two hundred μl of this mixture was then transferred to a fresh 6 ml polypropylene tube and spiked with 2 ml acetonitrile containing 25 ng/ml $^{13}C_9$-PFNA (Cambridge Isotope Laboratories, Inc., Andover, MA). The tube was vortexed for 20 minutes and then centrifuged for 3 minutes at 3500 rpm to precipitate proteins or other residue. Two hundred μl of the supernatant was then transferred to a 500 μL polypropylene autosampler vial and mixed with 200 μl of 2 C mM ammonium acetate

for HPLC/MS-MS analysis. Solutions were analyzed using an Agilent 1100 high-performance liquid chromatograph (Agilent Technology, Palo Alto, CA) coupled with an API 3000 triple quadrupole mass spectrometer (LC/MS-MS; Applied Biosystems, Foster City, CA). Ten μl of solution was injected onto a Luna C18(2) 3×50 mm, 5 μm column (Phenomenex, Torrance, CA) using a mobile phase consisting of 30% 2 mM ammonium acetate solution and 70% acetonitrile. Peak integrations and areas were determined using Analyst software (Applied Biosystems Version 1.4.1). For each analytical batch, matrix-matched calibration curves were prepared using mouse serum spiked with varying levels of PFNA (Aldrich, St. Louis, MO). For quality control, check standards were prepared by spiking large volumes of mouse serum at several arbitrary levels. Check standards were stored frozen and aliquots analyzed with each analytical set. In addition, control mouse serum samples were fortified at two or three levels in duplicate with known quantities of PFNA during the preparation of each analytical set. Duplicate fortified and several check standards were run in each analytical batch to assess precision and accuracy. The limit of quantitation (LOQ) was set as the lowest calibration point on the standard curve. Analytical batches were considered to be acceptable if matrix and reagent blanks had no significant PFNA peaks approaching the LOQ, the standard curve had a correlation coefficient > 0.98, and all standard curve points, fortified, and check samples were within 70%–130% of the theoretical and previously determined values, respectively.

2.4. Data Analysis. Maternal pregnancy, neonatal development, and necropsy data were analyzed in GraphPad Prism (version 4; San Diego, CA). Individual means (maternal data) or litter means (pup data) and standard errors were obtained by dose group and strain and analyzed by ANOVA. Pairwise *t*-tests were computed within ANOVA to compare individual treatment groups to relevant control groups within strain. A Bonferroni multiple-comparison adjustment was used when appropriate. Linear regression analysis was performed on liver data to detect dose-related trends. Pregnancy rate was analyzed using chi-square trend analysis. Litter loss is described as dams that had full litter resorption (FLR, uterine implants but no pups at birth) or whole litter loss (WLL, only dead pups at birth). Litter loss was examined for treatment effect using chi-square analysis. Serum data were analyzed in SAS for Windows v9.1 (SAS, Cary, NC). Analyses were performed separately for adult females and for pups. Adult females were further separated into pregnancy and lactation status (with live pups or with no live pups including nonpregnant and litter loss). A subset of dams matched with their pups was used to determine differences in levels of PFNA between dams and pups. Where variances were heterologous, data were log10 transformed to calculate means and standard errors and analyzed by ANOVA to investigate effects of treatment, strain (WT, KO), and block. When treatment differences were found by ANOVA, pairwise t-tests between treatment groups were calculated within each strain and separately by dams or pups, using Tukey-Kramer adjustment for multiple comparisons where appropriate.

3. Results

3.1. Maternal Pregnancy Outcome and Gestational Body Weight. Daily maternal body weight and maternal weight gain from GD 1 to GD 18 were not affected by gestational PFNA exposure. Implantation and total litter size (live and dead pups) at birth were not affected in either strain. However, the number of live pups at birth was significantly reduced in the WT strain at 1.1 ($P < .05$) and 2.0 ($P < .001$) mg/kg PFNA (Table 1) while being not significant at 1.5 mg/kg. Percent litter loss was not significantly altered in any dose group in KO or WT although there was a modest but insignificant increase in litter loss in the WT (Table 1). In each dose group in the KO, only 1 or 2 dams had FLR or WLL while, in the WT group, exposed to 2 mg/kg PFNA, 4 dams had FLR and 2 had WLL (35% litter loss). Most dams with FLR did not gain weight comparable to the pregnant dams that delivered litters, which suggests that FLR occurred early in gestation. Dams with WLL gained weight and carried to term, but it cannot be determined by our protocol whether these pups died prior to delivery or soon after delivery. Pregnancy rate, the percentage of plugged mice that had uterine implants, was reduced in treated KO groups ($P < .001$) but not in WT groups, suggesting that PFNA may have interfered with implantation when PPARα was not functional.

3.2. Pup Survival, Development, and Body Weight. The reduced viability of pups at birth in the WT at 1.1 and 2 mg/kg continued through the postnatal period. Survival of WT pups from birth to weaning (PND 21) was greatly reduced at 1.1 ($P < .05$) and 2 ($P < .001$) mg/kg PFNA (Figure 1). By PND 21, survival of pups in the WT 1.1 and 2.0 mg/kg groups was reduced to 36% and 31%, respectively. In contrast, survival was not affected in the KO at any dose.

Eye opening was used as a marker of postnatal development. The mean day of eye opening in the controls was PND 13.7 ± 0.3 in WT and PND 13.9 ± 0.2 in KO. The mean day of eye opening was significantly delayed at 2 mg/kg PFNA in the WT by two days, to PND 15.8 ± 0.2 ($P < .01$), but not at any other dose. In contrast, the mean day of eye opening was not affected at any dose in KO. The percent of eyes open on PNDs 13, 14, 15, and 16 was also significantly reduced in the WT at 2 mg/kg PFNA while being not affected in the KO (Figure 2).

Pup birth weight was not affected by any dose of PFNA in WT or KO, either in males or females (Table 2). Although pup body weight was not different among groups at birth, pup body weight was reduced in both male and female WT pups in the 2 mg/kg group at several time points during the postnatal period, beginning at PND 7 and including weaning (Figure 3). Weight gain during this period was reduced in WT female pups from 8.52 g in controls to 6.35 g in the 2 mg/kg group ($P < .001$), but not in male. In contrast, body weight and weight gain were not affected at any age at any dose level in the KO (Figure 3).

3.3. Liver Weight and Body Weight at PND21. Absolute liver weight was increased in a dose-dependent fashion in

Table 1: Effects of gestational administration (GD 1–18) of PFNA to wild type and PPARαKO mice on maternal weight and reproductive outcomes.

Strain	Dose (mg/kg/day)	No. of pregnant[a]	Maternal Weight Gain GD1–18[b] (g)	Maternal Weight GD18[b] (g)	No. of uterine implants	Total no. of Pups per litter[c] (live + dead)	No. of live pups per litter[c]	% Litter loss[d]	Pregnancy rate[e] (%)
WT	0	14	10.8 ± 0.98	34.4 ± 0.8	8.5 ± 0.6	7.1 ± 0.56	6.8 ± 0.70	14.3	53.8
	0.83	11	12.8 ± 0.81	35.0 ± 1.0	8.5 ± 0.6	6.8 ± 0.70	6.1 ± 0.82	9.1	47.8
	1.1	12	10.8 ± 0.57	33.6 ± 0.4	7.3 ± 0.5	5.6 ± 0.50	3.7 ± 0.37*	16.7	35.3
	1.5	14	12.6 ± 0.50	36.0 ± 0.5	8.4 ± 0.6	6.2 ± 0.54	4.7 ± 0.67	7.1	38.9
	2.0	17	13.2 ± 0.74	35.9 ± 0.9	7.8 ± 0.6	5.2 ± 0.54	3.1 ± 0.73**	35.3	47.2
KO	0	18	12.0 ± 0.49	35.9 ± 0.6	8.9 ± 0.4	7.8 ± 0.36	7.0 ± 0.41	11.1	75.0
	0.83	13	11.4 ± 0.73	34.6 ± 0.8	8.4 ± 0.6	7.2 ± 0.58	7.0 ± 0.54	7.7	65.0†
	1.1	14	11.5 ± 0.56	35.6 ± 0.6	9.2 ± 0.4	8.3 ± 0.49	7.8 ± 0.43	7.1	58.3†
	1.5	9	12.1 ± 0.87	35.5 ± 1.3	9.8 ± 0.6	8.5 ± 0.80	8.4 ± 0.84	11.1	20.9†
	2.0	16	11.0 ± 0.80	33.4 ± 0.9	8.1 ± 0.7	6.6 ± 0.62	6.4 ± 0.66	12.5	43.2†

Values are means ± SEM.
KO: PPARα knockout; WT: wild type; GD: gestational day.
[a]Pregnancy verified by presence of uterine implantation sites.
[b]Excludes adult females not pregnant or with full litter resorption.
[c]Number of pups on day of birth at first observation.
[d]Litter loss: uterine implants present but no pups (full litter resorption) or only dead pups (whole litter loss) at birth.
[e]Pregnancy rate: (# pregnant / # plug +)∗100.
$^*P < .05$, $^{**}P < .001$ by Bonferroni's test. $^\dagger P < .001$ by chi-square test for trend.

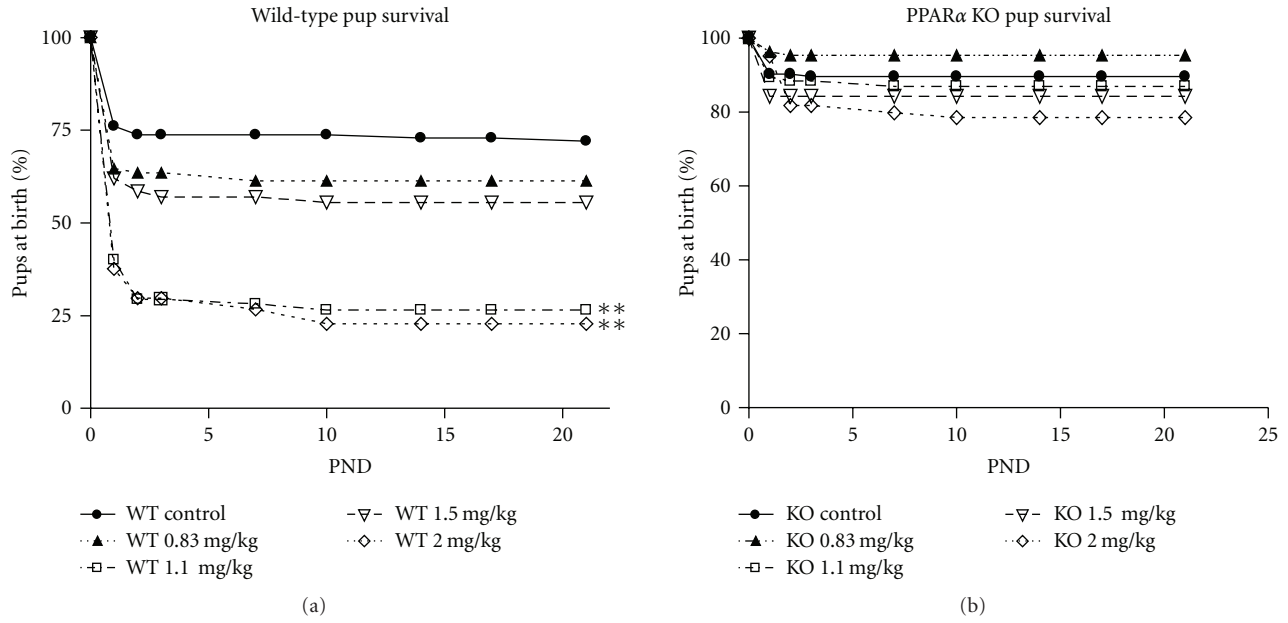

Figure 1: Effects of prenatal exposure to PFNA on survival of wild-type (WT) and PPARα knockout (KO) mouse pups. Data represent litter means ± SEM of the percent of the litter alive on postnatal days 0–3, 7, 10, 14, 17, and 21. Survival was reduced in WT pups by 1.1 and 2.0 mg/kg PFNA on GDs 1–18. Asterisks denote a significant difference ($P < .001$) found by ANOVA and Bonferroni's test for multiple comparisons. PND: postnatal day.

all PFNA-treated groups in WT adult females, regardless of prior pregnancy status. In KO adult females, however, liver weight was not affected by PFNA in dams but was increased in the 1.5 and 2.0 mg/kg groups in the nonpregnant adult (Table 3). In addition, among the nonpregnant adults, the dose dependent increase in liver weight was lower in KO compared to WT ($P < .0001$, by regression analysis). Similarly, relative liver weight was increased in a dose-dependent fashion in all treated groups in the WT ($P < .001$), regardless of pregnancy history, and in 1.1 mg/kg and higher

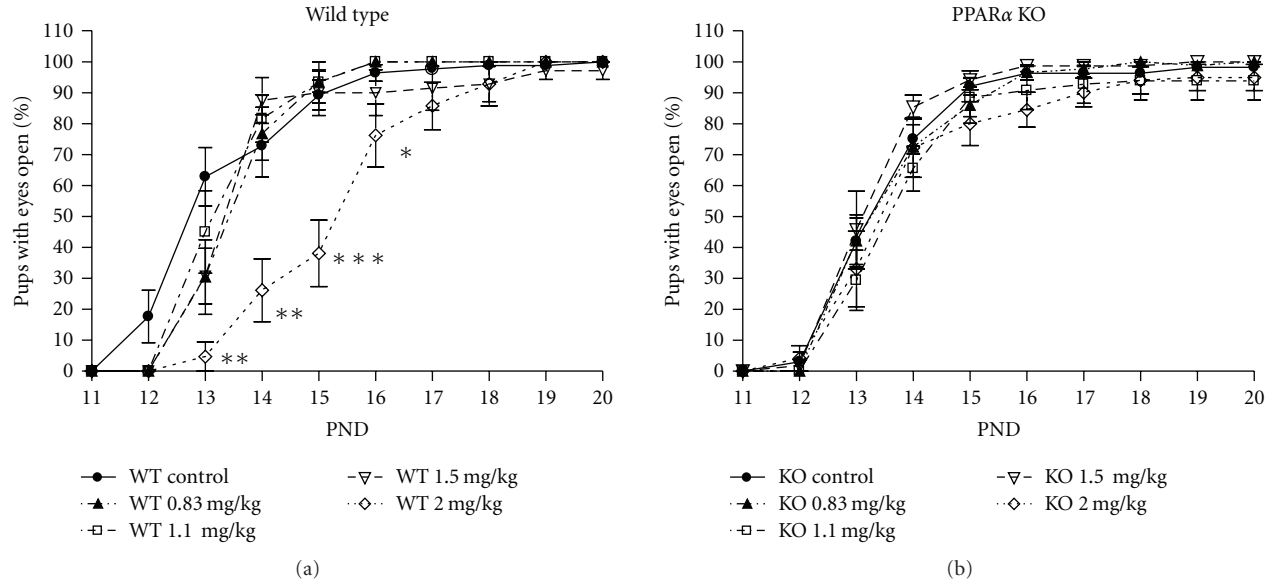

Figure 2: Effect of prenatal exposure to PFNA on the percent of eyes open on postnatal days 13–16 in wild-type (WT) and PPARα knockout (KO) mouse pups. Data represent litter means ± SEM of the percent of the litter with pups having both eyes fully open. A reduction in the percent of eyes open was found in the WT pups exposed to 2 mg/kg PFNA. Differences were found by ANOVA and Bonferroni's test for multiple comparisons. Asterisks denote a significant difference ($^*P < .05$, $^{**}P < .01$, $^{***}P < .0001$). PND: postnatal day.

Table 2: Birth weights of wild type and PPARα KO mouse pups after in utero exposure to PFNA on GD 1–18.

	Dose (mg/kg/day)	*n*	Male weight (g)	*n*	Female weight (g)
WT	0	11	1.28 ± 0.03	12	1.26 ± 0.03
	0.83	8	1.28 ± 0.03	10	1.30 ± 0.05
	1.1	10	1.29 ± 0.06	10	1.34 ± 0.06
	1.5	11	1.33 ± 0.03	12	1.32 ± 0.03
	2.0	9	1.41 ± 0.06	8	1.30 ± 0.05
KO	0	16	1.24 ± 0.03	16	1.19 ± 0.02
	0.83	12	1.28 ± 0.03	12	1.25 ± 0.03
	1.1	12	1.25 ± 0.04	13	1.20 ± 0.03
	1.5	8	1.20 ± 0.04	8	1.15 ± 0.04
	2.0	10	1.26 ± 0.03	14	1.29 ± 0.05

Values are litter means ± SEM. *n*: no. of litters. WT: wild type; KO: PPARα knockout; *n*: number of litters.

doses in the nonpregnant KO (Figure 4). In KO adults that had been pregnant, relative liver weight was unaffected. Body weight at necropsy was generally unaffected by dose or strain (Table 3). Absolute liver weight was increased in all PFNA dose groups in WT pups but was unaffected in KO. Relative liver weight was increased in all dose groups in WT pups but in only the highest dose group, 2 mg/kg, in KO (Figure 4). Body weight was not reduced in KO pups at any dose. Pup body weight was reduced in WT at 2 mg/kg only (Table 3).

3.4. Serum PFNA Levels. PFNA was detected in serum of all animals (Table 4). PFNA levels were significantly higher in PFNA-treated mice at every dose level compared to controls ($P < .0001$) and levels increased in a dose-dependent fashion. Serum PFNA levels were higher in adult females with no live pups (regardless of pregnancy) compared to adults with live pups by $P < .001$ (KO) and $P < .005$ (WT). PFNA levels were also higher in pups compared to their dams, based on a subset of dams matched to their existing pups at weaning (KO, $P < .0001$; WT, $P < .005$). In all dams with nursing pups, PFNA levels were lower in KO compared to WT ($P < .001$) while, in pups, PFNA levels were higher in KO compared to WT ($P < .0001$; Table 4).

4. Discussion

Perfluorononanoic acid (PFNA) has recently been shown to induce developmental toxicity and liver enlargement in mice [21], as do other perfluoroalkyl acids. The purpose of the current study was to determine whether these effects are dependent on PPARα, using the 129S/SvlmJ PPARα knockout (KO) mouse model. Gestational exposure to PFNA reduced neonatal survival and body weight through the weaning period, delayed eye opening, and increased absolute liver weight in the WT offspring at doses as low as 0.83 mg/kg/day. By contrast, these effects were not seen in KO offspring. These findings demonstrate that PFNA is a developmental toxicant and its effects are dependent on expression of PPARα.

This pattern of reduced survival, body weight, delayed development, and increased liver weight is common to most perfluoroalkyl acids (PFAAs) studied thus far. These effects have been reported in rodents for PFOA [30, 34], PFOS, [31, 35, 36], and PFNA [[21, 37], Das, 2010 #389], with a few specific differences that may be due to strain, dosing regimen, and the chain length and functional group of the PFAA. Such

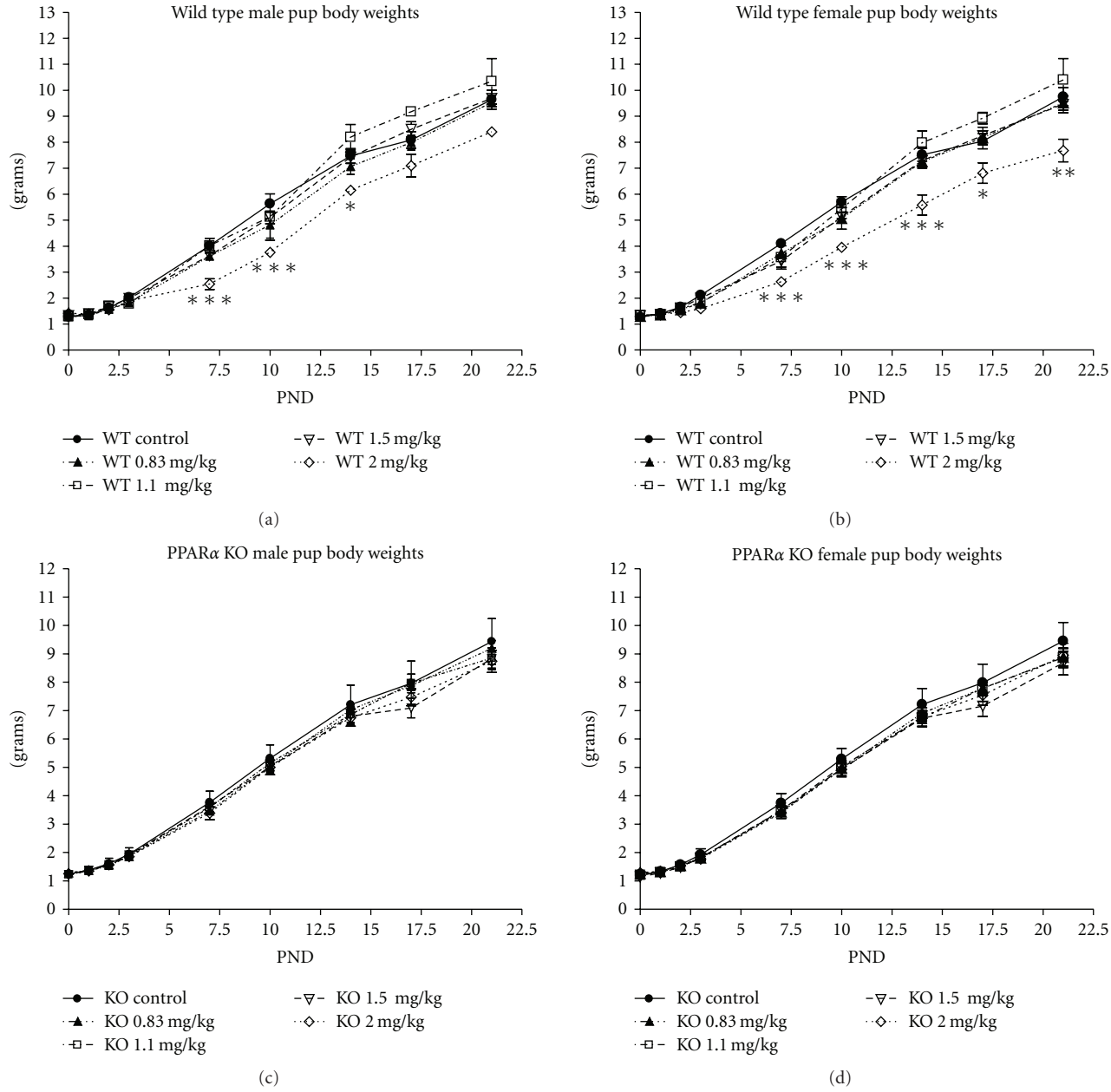

Figure 3: Effects of prenatal exposure to PFNA on postnatal body weights of wild-type (a, b) and PPARα knockout (c, d) male (a, c) and female (b, d) pups. Data represent litter means ± SEM on postnatal days 0–3, 7, 10, 14, 17, and 21. Body weights of WT pups were reduced by 2 mg/kg PFNA on postnatal days 7, 10, and 14, in male pups and days 7–21 in females. No effect on body weight was found in KO pups. Significant differences were found by ANOVA, and differences between groups were found by Bonferroni's test for multiple comparisons. Asterisks denote a significant difference (*$P < .05$ by t-test, ** $P < .01$, *** $P < .001$).

studies also obtained effects in offspring at dose levels that are not maternally toxic [30, 31, 34, 35, 38], as shown here. Also common to the current and previous studies, the liver was the most sensitive target tissue, with effects on liver weight seen in both WT dam and pup from the lowest dose level of PFNA used in the study, 0.83 mg/kg, and higher. Reduced survival, body weight, and delayed eye opening in pups were also sensitive endpoints, inducing effects at the next higher dose levels, 1.1 and/or 2.0 mg/kg. Survival and number of live pups at birth were compromised at 1.5 mg/kg, but the values did not reach statistical significance. The reason for this finding is unclear. The serum PFNA concentrations and the liver weight in the pups in this dose group were in the expected ranges for a linear dose-response curve, suggesting proper dose preparation and administration for 1.5 mg/kg. In addition, the "n" of 12 litters in this treatment group was comparable to that of other dose groups, so it seems unlikely that the outcome is related to a low statistical

Table 3: Liver and body weights (grams) of wild type and PPARα-KO adult females and pups at necropsy (PND 21) after exposure to PFNA on GD 1–18.

Strain	Dose (mg/kg/ day)	Adult females (NP)		Adult females (P)		Pups (sexes combined)	
		Liver Weight	Body Weight	Liver Weight	Body Weight[a]	Liver Weight	Body Weight
WT	0	0.86 ± 0.03	22.7 ± 0.42	1.52 ± 0.87	27.3 ± 0.58	0.381 ± 0.02	9.62 ± 0.36
	0.83	1.49 ± 0.04**	22.9 ± 0.48	1.65 ± 0.07	26.4 ± 0.56	0.551 ± 0.03**	9.79 ± 0.30
	1.1	1.80 ± 0.04**	24.0 ± 0.43	1.91 ± 0.10*	26.2 ± 0.72	0.649 ± 0.05**	10.34 ± 0.61
	1.5	1.86 ± 0.05**	23.0 ± 0.56	2.24 ± 0.05**	28.2 ± 0.66	0.608 ± 0.02**	9.47 ± 0.23
	2.0	2.18 ± 0.04**	24.2 ± 0.36	2.51 ± 0.07**	27.5 ± 0.70	0.518 ± 0.01**	7.56 ± 0.42**
KO	0	0.99 ± 0.04	24.8 ± 0.74	1.88 ± 0.06	29.1 ± 0.32	0.417 ± 0.01	9.35 ± 0.19
	0.83	1.20 ± 0.04	24.7 ± 1.00	1.77 ± 0.06	28.3 ± 0.38	0.421 ± 0.01	9.16 ± 0.28
	1.1	1.17 ± 0.07	23.2 ± 0.54	2.02 ± 0.05	29.6 ± 0.40	0.429 ± 0.02	9.18 ± 0.26
	1.5	1.45 ± 0.04**	23.8 ± 0.41	1.74 ± 0.16	26.2 ± 1.32*	0.422 ± 0.02	8.51 ± 0.34
	2.0	1.53 ± 0.04**	23.4 ± 0.28	1.96 ± 0.10	28.0 ± 0.77	0.489 ± 0.03	8.98 ± 0.40

Values are means ± SEM.
Pup weights were on 2 pups per litter. NP: not pregnant; P: pregnant; WT: wild type; KO: PPARα knockout; Wt: weight.
NP includes those with full litter resorption and no weight gain; P includes those who gave birth whether pups were live or dead.
[a]$P < .05$ compared to NP females body weight. *$P < .05$, **$P < .001$ compared to controls within column and strain. See text for other comparisons.

Table 4: Serum PFNA concentrations at weaning in PPARα-KO and WT Adult female mice and offspring exposed to PFNA on GD 1–18.

Strain	Dose (mg/kg/day)	Adult females with no live pups		Adult females with live pups		Pups (sexes combined)	
		n	PFNA (μg/ml)	*n*	PFNA (μg/ml)	*n* litters	PFNA (μg/ml)
WT	0	14	0.067 ± 0.005	12	0.022 ± 0.004	9	0.033 ± 0.008
	0.83	13	28.5 ± 1.22[a]	10	8.91 ± 1.51[a]	8	9.60 ± 9.37[a]
	1.1	26	39.7 ± 1.26[a]	10	23.2 ± 2.57[a]	5	15.7 ± 1.42[a]
	1.5	23	48.4 ± 1.54[a]	13	21.0 ± 3.01[a]	10	17.5 ± 1.15[a]
	2.0	26	64.0 ± 2.46[a]	11	35.3 ± 3.90[a]	7	25.3 ± 2.70[a]
KO	0	9	0.048 ± 0.008	16	0.016 ± 0.001	16	0.068 ± 0.027
	0.83	8	38.4 ± 2.34[a]	11	2.76 ± 0.172[a]	12	15.2 ± 1.01[a]
	1.1	11	53.9 ± 2.51[a]	13	4.17 ± 0.310[a]	12	19.4 ± 0.69[a]
	1.5	37	72.1 ± 2.91[a]	8	11.8 ± 5.71[a]	7	26.4 ± 1.39[a]
	2.0	23	83.4 ± 2.93[a]	15	22.6 ± 5.69[a]	12	38.4 ± 1.80[a]

Values are means ± SEM or litter means ± SEM. Serum was collected from all adult females and from 2 pups per litter at 23 days post dose.
WT: wild type; KO: PPARα knockout.
[a] Significantly different from control values by $P < .0001$. See text for more statistical comparisons.

power. Thus, the lack of consistent effect on survival cannot be explained and may simply reflect biological variability. Nonetheless, all developmental endpoints were clearly PPARα dependent. The dependence of the developmental effects of PFNA on PPARα is not unique, as this has also been demonstrated previously for PFOA [30]. However, not all PFAAs depend upon PPARα to induce developmental effects. The developmental effects of PFOS, for example, were not found to be dependent on PPARα [31]. This may be due to the sulfonated head group of PFOS, and thus PPARα dependence may be a feature of the perfluorocarboxylic acids.

Mode of action differences between the perfluorinated carboxylic acids, PFOA and PFNA, and the sulfonate PFOS may also be evident in the pattern of neonatal loss observed following exposure to these compounds. PFNA exposure in WT mice resulted in a drastically reduced number of viable pups at birth with a continued loss of pups within the first few days, followed by a gradual loss until PND 10. Similarly, PFOA induced a sudden loss of viable pups within the first few days of life, with a gradual loss over 10 days in CD-1 mice [38] and 14 days in the 129S/SvlmJ strain [30]. In contrast to our study, PFNA in CD-1 mice induced a gradual loss of pups over the course of 12 days with no significant loss at birth [21]. This difference may be due to the increased sensitivity of the 129S/SvlmJ strain. Although survival curves for PFOA and PFNA can follow a course of up to 10–14 days, *in utero* exposure to PFOS results in a sudden loss of viability in pups within the first few hours after birth through postnatal day 2 in the rat [35, 36]. These pups were observed to be in respiratory distress and displayed poor inflation of the lungs [36, 39] although the precise mechanism has not been found. This two day loss of pups after exposure to PFOS was observed in the 129S/SvlmJ strain as well, and only in KO did a few more die as late as PND 10 [31]. Therefore, in neonates, PFNA may be utilizing the same mechanism of action as other perfluorinated carboxylates while sulfonates such as PFOS utilize another.

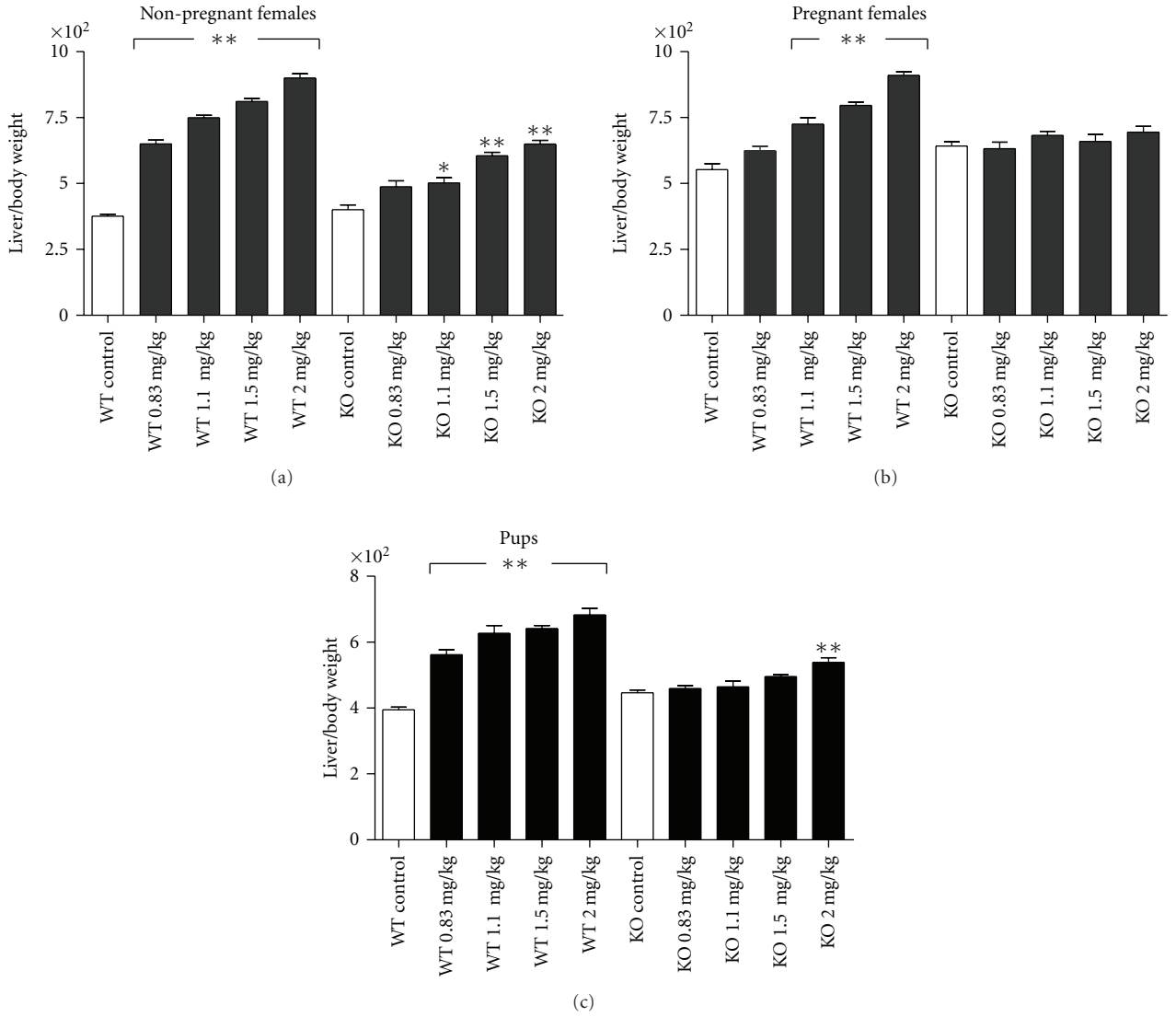

Figure 4: Effects of gestational exposure to PFNA on relative liver weight of the wild-type (WT) and PPARα knockout (KO) nonpregnant adult female (a) dams (b) and pups (c) Measurements were taken on all individual adult females and on two pups per litter at weaning (i.e., 23 days after last dose or postnatal day 21). Data represent means or litter means ±SEM. Relative liver weight was calculated as the absolute liver weight/body weight ×100 for each data point. Relative liver weight was increased by PFNA exposure in both pregnant and non-pregnant adults and pups in all treated groups in the WT while only in the nonpregnant adult KO at 1.1 to 2 mg/kg and in the KO pup at 2.0 mg/kg. Significant differences were found by ANOVA, and differences between groups were found by Bonferroni's test for multiple comparisons. Asterisks denote significant differences compared to controls ($^*P < .05$, ** $P < .001$).

In the liver, there appear to be PPARα-independent as well as PPARα-dependent events in response to PFNA. PFNA was found to increase relative and absolute liver weight in the WT adult, but to a lesser extent in the nonpregnant KO, and not at all in the pregnant KO adult. The lack of effect on liver weight in the pregnant KO may suggest that the effects of PFNA on liver weight in adult KO mice are modest and were masked by the increase in liver weight due to pregnancy. The attenuated response in the KO liver compared to the WT liver is more obvious in the pup and may imply a separate, less efficient mechanism independent of PPARα. Similarly, less robust effects on liver weight in KO compared to WT mice were observed after exposure to PFOA [30]. Histopathological examination of those livers revealed a difference in histology of treated KO livers compared to treated WT livers [40], suggesting a different mechanism in KO mice. Other pathways suggested have included constitutive androstane receptor (CAR) and pregnane X receptor (PXR) [41–43], both present in humans. Therefore, PFNA may primarily utilize PPARα to increase liver weight while relying upon other pathways in the absence of PPARα. Involvement of PPARα in the liver may be a mechanism utilized by other PFAAs, since perfluorobutyrate also increased liver weight and induced hepatocyte hypertrophy dependent on

PPARα [44]. Relevance of the PPARα mechanism to humans has been criticized primarily based on the lower number of these receptors in the liver of human versus mouse. However, PPARα is implicated here in the developmental effects of PFNA as well, and the etiology of PPARα in other tissues of the embryo, fetus and neonate of the human and the mouse that are involved in gross development has not been fully determined. Therefore, the possibility of relevance of PPARα to a human response to PFNA cannot be dismissed.

The levels of PFNA in the serum of pups, nursing dams, and adult females with no pups illustrate some interesting findings. First, the dose-dependent serum levels of PFNA in all groups of animals reflect the dose-dependent effects observed in dams and pups. Second, the effects observed in WT pups were not due to higher concentrations of PFNA in their system, since serum levels of PFNA were actually lower in WT pups than in KO pups at all doses. Conversely, the general lack of developmental effects in KO pups was not due to impaired pharmacokinetic distribution of PFNA to the pup. Another important observation is the possibility of substantial transfer of PFNA from dam to pup through milk. PFNA can enter milk, as evidenced by the finding of PFNA in the milk of humans [45–47], rats [48], and mice [49]. The lower serum PFNA levels in lactating dams compared to nonlactating adult females at weaning suggest an elimination of PFNA from the dams through placental transfer and through the milk. In addition, PFNA levels were elevated in pups compared to their mothers. This has also been reported for PFOA, in which an increased body burden was observed in the pups from birth to postnatal day 8 [49]. However, the contribution of placental versus lactational transfer of PFNA cannot be determined by the design of this study. Serum levels of PFNA in this mouse model were much higher than those of humans [7] but were measured to compare with the physiological effects observed and not to compare to human levels.

PFNA was found in this study to be a liver and developmental toxicant comparable in strength to other PFAAs, as adverse responses were elicited at maternal doses as low as 0.83 mg/kg. In the CD-1 mouse, PFNA appears to be more potent than PFOA. PFNA reduced CD-1 pup survival at 5 mg/kg/day, compared to10 mg/kg/day by PFOA, and delayed eye opening at 3 mg/kg/day compared to 5 mg/kg/day by PFOA [21, 34, 37, 38]. *In vitro* analysis of PPARα activation shows PFNA to be more potent than PFOA as well [28]. The 129S strain used in the current study was used as an animal model for investigating mechanisms of action rather than for relative potency, as toxicity and PBPK data are lacking and this strain appears to be more sensitive to PFAAs. It is also clear that PFNA is more potent than PFOS. PFOS induced a 50% reduction in survival in CD-1 offspring at 10 mg/kg/day [35] and at 8.5 mg/kg/day in 129S/SvlmJ mice [31] whereas PFNA reduced survival at 1.1 mg/kg/day in the current study or 5 mg/kg in CD-1 mice. Given the lower activity of the sulfonated PFAA compared to the carboxylated PFAA on PPARα *in vitro* [28], lower potency *in vivo* may be expected for other sulfonated PFAAs as well.

5. Conclusion

In summary, PFNA is a developmental toxicant in mice, and the developmental effects are dependent upon expression of PPARα. The general pattern of effects observed in the mouse after gestational PFNA exposure mirrors the effects of other PFAAs, most closely that of PFOA. In addition, the differential response to PFNA in the livers of WT and KO adult females suggests a PPARα-dependent mode of action for increased liver weight, although additional pathways and mechanisms appear to be involved.

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Research Article

Gene Expression Profiling in Wild-Type and PPARα-Null Mice Exposed to Perfluorooctane Sulfonate Reveals PPARα-Independent Effects

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Perfluorooctane sulfonate (PFOS) is a perfluoroalkyl acid (PFAA) and a persistent environmental contaminant found in the tissues of humans and wildlife. Although blood levels of PFOS have begun to decline, health concerns remain because of the long half-life of PFOS in humans. Like other PFAAs, such as, perfluorooctanoic acid (PFOA), PFOS is an activator of peroxisome proliferator-activated receptor-alpha (PPARα) and exhibits hepatocarcinogenic potential in rodents. PFOS is also a developmental toxicant in rodents where, unlike PFOA, its mode of action is independent of PPARα. Wild-type (WT) and PPARα-null (Null) mice were dosed with 0, 3, or 10 mg/kg/day PFOS for 7 days. Animals were euthanized, livers weighed, and liver samples collected for histology and preparation of total RNA. Gene profiling was conducted using Affymetrix 430_2 microarrays. In WT mice, PFOS induced changes that were characteristic of PPARα transactivation including regulation of genes associated with lipid metabolism, peroxisome biogenesis, proteasome activation, and inflammation. PPARα-independent changes were indicated in both WT and Null mice by altered expression of genes related to lipid metabolism, inflammation, and xenobiotic metabolism. Such results are similar to studies done with PFOA and are consistent with modest activation of the constitutive androstane receptor (CAR), and possibly PPARγ and/or PPARβ/δ. Unique treatment-related effects were also found in Null mice including altered expression of genes associated with ribosome biogenesis, oxidative phosphorylation, and cholesterol biosynthesis. Of interest was up-regulation of *Cyp7a1*, a gene which is under the control of various transcription regulators. Hence, in addition to its ability to modestly activate PPARα, PFOS induces a variety of PPARα-independent effects as well.

1. Introduction

Perfluoroalkyl acids (PFAAs) are stable man-made perfluorinated organic molecules that have been utilized since the 1950s in the manufacture of a variety of industrial and commercial products such as fire fighting foams, fluoropolymers for the automobile and aerospace industry, paper food packaging, stain-resistant coatings for carpet and fabric, cosmetics, insecticides, lubricants, and nonstick coatings for cookware. One such PFAA, perfluorooctane sulfonate (PFOS), was identified nearly a decade ago as a persistent organic pollutant which could also be found in the tissues of wildlife throughout the globe [2]. Since that time, a number of perfluorinated sulfonic and carboxylic

Table 1: Average body weight and liver weight of control and PFOS-treated mice on the day of tissue collection.[1]

Dose group	WT			Null		
	Body weight	Total liver weight	Relative liver weight	Body weight	Total liver weight	Relative liver weight
0 mg/kg	28.3 ± .0	1.21 ± 0.17	0.043 ± 0.014	30.3 ± 1.3	1.04 ± 0.06	0.034 ± 0.003
3 mg/kg	26.2 ± 1.5	1.12 ± 0.18	0.043 ± 0.002	28.0 ± 1.2	1.20 ± 0.05	0.043 ± 0.001
10 mg/kg	31.4 ± 1.5	1.98 ± 0.11*	0.062 ± 0.003*	30.2 ± 1.7	1.48 ± 0.16*	0.049 ± 0.012*

[1] Data are mean ± SE, *Significantly different than control ($P \leq .05$).

Table 2: Number of fully annotated genes altered by PFOS, PFOA[1], or Wy-14,643[1] in wild-type and PPARα-null mice ($P \leq .0025$)[2].

	PFOS		PFOA	Wy 14,643
	3 mg/kg/day	10 mg/kg/day	3 mg/kg	50 mg/kg/day
Wild-type	81	906	879	902
PPARα-null	630	808	176	10

[1] From Rosen et al. (2008), [2] Based on Ingenuity Pathways Analysis database.

acids of varying chain length have been shown to be persistent and ubiquitous environmental contaminants. Some of these compounds are also commonly identified in the tissues of humans and wildlife with the 8-carbon PFAAs, PFOS and perfluorooctanoic acid (PFOA), being the most frequently reported in biomonitoring studies (for reviews, see [3, 4]). In recent years, blood levels of PFOS and PFOA have gradually begun to decline in the general population [5, 6]. This is due in part to a production phase out of PFOS by its principal U.S. manufacturer as well as a commitment by key manufacturers of perfluorinated chemicals to reduce the product content and emissions of PFOA, and related chemistries, under the EPA 2010/2015 PFOA Stewardship Program (http://www.epa.gov/oppt/pfoa/pubs/stewardship/index.html). Nevertheless, certain PFAAs are likely to remain of concern for years to come due to their environmental persistence and long biological-half lives [7].

PFOS and PFOA are associated with toxicity in laboratory animals at blood levels that are approximately 2-3 orders of magnitude above those normally observed in humans. This includes hepatomegaly and liver tumors in rats and mice as well as pancreatic and testicular tumors in rats (for review see [4]). Teratogenic activity has also been observed in rats and mice, however, such findings have been limited to maternally toxic doses of PFOS [8], whereas, both PFOS and PFOA have been shown to alter growth and viability of rodent neonates at lower doses [4]. Recent epidemiologic data suggests that typical exposures to these compounds may alter fetal growth and fertility in humans [9–13]. These studies, however, lack consistency with regard to either compound activity or measured end point; therefore, alternative explanations for such findings have been suggested [14]. Moreover, a recent study of individuals exposed to PFOA in drinking water at levels that were approximately two orders of magnitude higher than the general population did not show an effect on average birth weight or the incidence of low birth weight infants [15].

The mode of action related to PFAA toxicity in rodents is not fully understood. As a class of chemicals, PFAAs activate peroxisome proliferator-activated receptor alpha (PPARα) [16–18], and chronic activation of this nuclear receptor is thought to be responsible for the liver enlargement and hepatic tumor induction found in laboratory animals [19]. However, activation of PPARα is not thought to be a relevant mode of action for hepatic tumor formation in humans [20–25], although this assumption has been challenged recently [26]. This does not, however, rule out the possibility that certain PFAAs could have an adverse effect on development since activation of PPARα has been shown to play a role in PFOA-induced neonatal loss in mice [27]. In addition, PPARα-independent modes of action are also likely for various PFAAs. Unlike prototypical activators of PPARα, such as, the fibrate class of pharmaceuticals, PFOA can induce fatty liver in wild-type mice [28]. PFOA can also induce hepatomegaly in PPARα-null mice [27, 29, 30] and is capable of activating the constitutive androstane receptor (CAR) [31–33]. Moreover, PFOS can induce neonatal toxicity in the PPARα-null mouse [34].

In the current study, we used global gene expression profiling to assess the transcriptional changes induced by PFOS in the liver of wild-type and PPARα-null mice. The data were compared to results previously published by our group for PFOA and Wy-14,643, a commonly used agonist of PPARα [1]. Our goal was to identify both PPARα-dependent and independent changes induced by PFOS.

2. Materials and Methods

2.1. Animals and Dosing. Studies were approved by the U.S. EPA ORD/NHEERL Institutional Animal Care and Use Committee. The facilities and procedures used followed the recommendations of the 1996 NRC "Guide for the Care and Use of Laboratory Animals," the Animal Welfare Act, and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

PPARα-null (Null) mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J, stock no. 003580) and wild-type (WT) mice (129S1/SvlmJ, stock no. 002448) were initially purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained as an inbred

Table 3: Average fold change for genes related to lipid metabolism in wild-type and PPARα-null male mice following a seven-day exposure to Wy-14,643[1], PFOA[1], or PFOS.

Symbol	Gene name	Entrez no.	WT				Null		
			Wy14,643 50 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg
ACAA1	acetyl-CoA acyltransferase 1	113868	1.89	2.92	1.61	2.10**	1.22	1.37	1.53*
ACAA1B	acetyl-CoA acyltransferase 1B	235674	2.38	2.70	1.49	1.40**	3.00	1.09	1.19*
ACAD10	acyl-CoA dehydrogenase, member 10	71985	1.51	2.39	−1.18	1.38**	−1.01	1.05	1.20*
ACADL	acyl-CoA dehydrogenase, long chain	11363	3.03	2.86	1.40	1.68**	2.50	1.34	1.59**
ACADM	acyl-CoA dehydrogenase, C-4 to C-12	11364	1.70	1.30	1.21	1.31**	1.06	1.11	1.10
ACADS	acyl-CoA dehydrogenase, C-2 to C-3	66885	1.03	1.52	1.22	1.31*	−1.13	−1.12	−1.08
ACADSB	acyl-CoA dehydrogenase, short/branched	66885	−1.56	−1.64	−1.04	−1.39**	−1.26	1.00	−1.23
ACADVL	acyl-CoA dehydrogenase, very long chain	11370	1.92	1.80	1.44	1.49**	1.16	1.04	1.12
ACAT1	acetyl-CoA acetyltransferase 1	101446	−1.01	1.10	1.45	1.36*	−1.55	−1.05	−1.17
ACAT2	acetyl-CoA acetyltransferase 2	110460	2.59	1.68	1.14	1.34*	1.26	1.58	1.69**
ACOT1	acyl-CoA thioesterase 1	26897	19.48	73.06	3.27	6.82**	2.95	1.53	2.02
ACOT3	acyl-CoA thioesterase 3	171281	2.55	32.83	2.42	6.41**	−1.59	1.46	1.86
ACOT2	acyl-CoA thioesterase 2	171210	3.83	19.29	1.91	7.32**	1.78	1.25	1.52
ACOX1	acyl-CoA oxidase 1	11430	5.65	7.17	1.23	1.49**	1.51	1.30	1.29**
ACSL1	acyl-CoA synthetase long-chain member1	14081	1.34	2.36	1.28	1.36**	1.01	1.31	1.30
ACSL3	acyl-CoA synthetase long-chain member3	74205	2.25	1.90	1.28	1.69**	1.11	1.77	1.63
ACSL4	acyl-CoA synthetase long-chain member4	50790	1.95	2.00	1.03	1.42*	1.51	1.34	1.29
ACSL5	acyl-CoA synthetase long-chain member5	433256	3.06	2.76	1.24	1.31**	1.38	1.23	1.28
ALDH1A1	aldehyde dehydrogenase 1, member A1	11668	1.56	1.59	1.07	1.12**	1.22	1.16	1.17
ALDH1A7	aldehyde dehydrogenase 1, A7	26358	1.83	1.86	1.12	1.24*	1.55	1.26	1.35
ALDH3A2	aldehyde dehydrogenase 3, member A2	11671	3.65	7.72	2.10	3.80**	2.30	1.73	2.20**
ALDH9A1	aldehyde dehydrogenase 9, member A1	56752	1.80	1.91	1.27	1.50**	1.21	1.05	1.11*
CPT1B	carnitine palmitoyltransferase 1B (muscle)	12896	2.29	1.50	1.23	2.69**	−1.00	1.13	1.11
CPT2	carnitine palmitoyltransferase II	12896	1.33	2.54	1.58	2.03**	1.44	1.15	1.34
CYP4A14	cytochrome P450, 4, a, polypeptide 14	13119	75.38	103.48	11.26	12.28**	12.75	−1.09	2.22
DCI	dodecenoyl-CoA delta isomerase	13177	2.91	4.55	1.90	2.38**	1.99	1.04	1.38*
ECH1	enoyl CoA hydratase 1, peroxisomal	51798	3.27	5.23	1.93	2.49**	2.10	1.16	1.39

Table 3: Continued.

Symbol	Gene name	Entrez no.	WT Wy14,643 50 mg/kg	WT PFOA 3 mg/kg	WT PFOS 3 mg/kg	WT PFOS 10 mg/kg	Null PFOA 3 mg/kg	Null PFOS 3 mg/kg	Null PFOS 10 mg/kg
EHHADH	enoyl-CoA, hydratase	74147	27.89	22.11	2.37	4.34**	1.37	1.32	1.52*
FABP1	fatty acid binding protein 1, liver	14080	−1.27	1.02	1.11	1.24**	1.25	−1.09	−1.23
HADHA	Trifunctional protein, alpha unit	97212	2.13	2.95	1.37	1.65**	1.01	1.06	1.02
HADHB	Trifunctional protein, beta unit	231086	2.33	3.43	1.37	1.60**	1.08	−1.15	−1.28*
HSD17B4	hydroxysteroid (17-beta) dehydrogenase4	15488	2.03	2.56	1.34	1.45**	−1.13	1.12	1.20*
SLC27A1	solute carrier 27, member 1	26457	9.14	8.22	−1.02	1.14*	−1.57	1.04	1.04
SLC27A2	solute carrier 27, member 2	26458	1.48	1.80	1.19	1.16**	1.33	1.10	1.05
SLC27A4	solute carrier 27, member 4	26569	1.87	1.91	1.04	1.31**	−1.03	1.09	1.07

[1] From Rosen et al. (2008),
*Significantly different than control ($P \leq .03$),
**Significantly different than control ($P \leq .0025$)

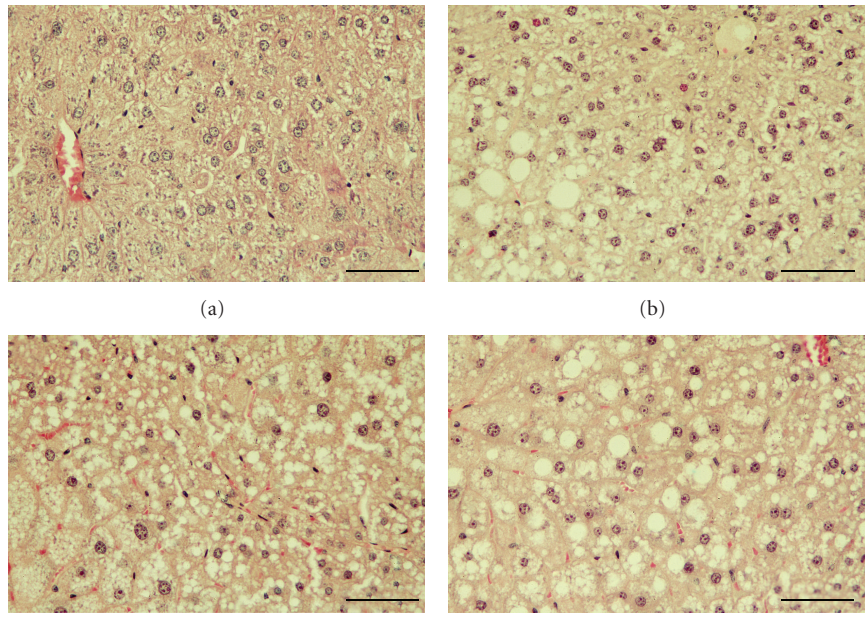

Figure 1: Hematoxylin-and eosin-stained tissue sections from control and PFOS treated mice. Control WT and Null mice are shown in panels (a) and (b), respectively. WT and null mice treated with 10 mg/kg/day PFOS are shown in panels (c) and (d), respectively. Vacuole formation was observed in sections from treated WT mice, and in sections from control and treated Null mice. Mice exposed to 3 mg/kg/day PFOS were similar to controls (data not shown). Bar = 50 μm.

colony on the 129/Sv background at the U.S. EPA, Research Triangle Park, NC. Animals were housed 5 per cage and allowed to acclimate for a period of one week prior to the conduct of the study. Food (LabDiet 5P00 Prolab RHM3000, PMI Nutrition International, St. Louis, MO) and municipal tap water were provided *ad libitum*. Animal facilities were controlled for temperature (20–24°C), relative humidity (40%–60%), and kept under a 12 hr light-dark cycle. The experimental design matched that of our previous study [1]. PPARα-null and wild-type male mice at 6–9 months of age were dosed by gavage for 7 consecutive days with either 0, 3, or 10 mg/kg PFOS (potassium salt, catalog no. 77282, Sigma Aldrich, St, Louis, MO) in 0.5% Tween 20. Five biological replicates consisting of individual animals

Table 4: Average fold change for genes related to proteasome biogenesis in wild-type and PPARα-null male mice following a seven-day exposure to Wy-14,643[1], PFOA[1], or PFOS.

Symbol	Gene name	Entrez no.	WT Wy14,643 50 mg/kg	WT PFOA 3 mg/kg	WT PFOS 3 mg/kg	WT PFOS 10 mg/kg	Null PFOA 3 mg/kg	Null PFOS 3 mg/kg	Null PFOS 10 mg/kg
PSMA1	proteasome unit, alpha type, 1	26440	1.61	1.38	1.15	1.31*	1.17	−1.29	−1.34
PSMA2	proteasome unit, alpha type, 2	19166	−1.46	−1.15	1.09	1.23**	−1.34	−1.20	−1.07
PSMA3	proteasome unit, alpha type, 3	19167	1.33	1.22	1.12	1.14	1.28	−1.13	−1.17
PSMA4	proteasome unit, alpha type, 4	26441	1.19	1.32	1.10	1.19*	1.01	−1.04	1.05
PSMA5	proteasome unit, alpha type, 5	26442	1.67	1.59	1.12	1.26**	1.15	−1.12	1.09
PSMA6	proteasome unit, alpha type, 6	26443	1.20	1.29	1.14	1.24**	1.06	−1.14	−1.06
PSMA7	proteasome unit, alpha type, 7	26444	1.47	1.60	1.23	1.53**	1.23	−1.12	1.11
PSMB1	proteasome unit, beta type, 1	19170	1.09	1.29	1.07	1.28*	1.04	−1.17	1.13*
PSMB10	proteasome unit, beta type, 10	19171	−1.42	−1.48	−1.25	−1.19	−1.57	−1.14	−1.21**
PSMB2	proteasome unit, beta type, 2	26445	1.33	1.48	1.05	1.31**	1.02	−1.20	1.05
PSMB3	proteasome unit, beta type, 3	26446	1.22	1.47	1.21	1.36**	1.04	−1.37	−1.20
PSMB4	proteasome unit, beta type, 4	19172	1.59	1.65	1.27	1.55**	1.22	−1.12	1.09
PSMB5	proteasome unit, beta type, 5	19173	1.34	1.74	1.04	1.24**	1.02	−1.15	1.03
PSMB6	proteasome unit, beta type, 6	19175	1.54	1.83	1.08	1.24*	1.19	−1.23	−1.09
PSMB7	proteasome unit, beta type, 7	19177	1.46	1.33	1.07	1.15**	1.13	−1.17	−1.09
PSMB8	proteasome unit, beta type, 8	16913	−1.61	−2.00	−1.44	−1.51	−1.38	−1.23	−1.45**
PSMB9	proteasome unit, beta type, 9	16912	1.24	−1.12	−1.31	−1.09	−1.10	−1.11	−1.30**
PSMC1	proteasome 26S unit, ATPase, 1	19179	1.44	1.00	1.19	1.15*	1.11	−1.06	1.01
PSMC6	proteasome 26S unit, ATPase, 6	67089	1.18	1.21	1.09	−1.02	1.07	1.14	−1.16
PSMD1	proteasome 26S unit, non-ATPase, 1	70247	1.20	1.22	1.15	1.25**	1.09	1.03	1.15
PSMD11	proteasome 26S unit, non-ATPase, 11	69077	1.56	1.38	1.09	1.26*	−1.17	1.16	1.32
PSMD12	proteasome 26S unit, non-ATPase, 12	66997	1.34	1.27	1.10	1.14	1.20	−1.03	1.04
PSMD13	proteasome 26S unit, non-ATPase, 13	23997	1.21	1.38	1.14	1.26*	−1.03	−1.38	−1.42**
PSMD14	proteasome 26S unit, non-ATPase, 14	59029	−1.39	−1.42	1.17	1.31*	1.31	1.01	1.17
PSMD2	proteasome 26S unit, non-ATPase, 2	21762	1.34	1.32	1.14	1.24*	1.10	1.09	1.30**

Table 4: Continued.

Symbol	Gene name	Entrez no.	Wy14,643 50 mg/kg	PFOA 3 mg/kg	WT PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	Null PFOS 3 mg/kg	PFOS 10 mg/kg
PSMD3	proteasome 26S unit, non-ATPase, 3	22123	−1.35	−1.19	1.17	1.29*	1.08	1.04	1.22*
PSMD4	proteasome 26S unit, non-ATPase, 4	19185	1.31	1.92	1.19	1.38**	1.03	−1.07	1.17*
PSMD6	proteasome 26S unit, non-ATPase, 6	66413	1.17	1.33	1.10	1.14*	1.07	−1.06	1.04
PSMD7	proteasome 26S unit, non-ATPase, 7	17463	1.13	1.27	1.13	1.24*	1.02	−1.19	−1.22*
PSMD8	proteasome 26S unit, non-ATPase, 8	57296	1.68	1.24	1.03	1.30**	1.16	−1.15	−1.00
PSME1	proteasome activator unit 1	19186	1.22	−1.00	−1.05	1.32**	1.27	−1.10	−1.09
VCP	valosin−containing protein	269523	1.40	1.49	1.04	1.12	1.07	1.13	1.21**

[1] From Rosen et al. (2008),
*Significantly different than control ($P \leq .03$),
**Significantly different than control ($P \leq .0025$).

Table 5: Average fold change for genes related to peroxisome biogenesis in wild-type and PPARα-null male mice following a seven-day exposure to Wy-14,643[1], PFOA[1], or PFOS.

Symbol	Gene name	Entrez no.	Wy14,643 50 mg/kg	PFOA 3 mg/kg	WT PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	Null PFOS 3 mg/kg	PFOS 10 mg/kg
PECI	peroxisome D3, D2-enoyl-CoA isomerase	23986	1.73	3.15	1.61	1.87**	1.96	1.42	1.57**
PEX1	peroxisomal biogenesis factor 1	71382	1.25	1.84	1.07	1.21**	−1.02	1.10	1.14*
PEX11A	peroxisomal biogenesis factor 11 alpha	18631	1.80	6.71	1.70	2.99**	1.04	−1.09	−1.11
PEX12	peroxisomal biogenesis factor 12	103737	1.07	1.36	1.11	1.17*	1.09	1.17	1.30*
PEX13	peroxisomal biogenesis factor 13	72129	1.04	1.58	1.01	1.09	1.02	1.09	1.16*
PEX14	peroxisomal biogenesis factor 14	56273	1.06	1.24	1.03	1.25*	1.03	1.05	1.13
PEX16	peroxisomal biogenesis factor 16	18633	1.51	1.44	1.13	1.33**	−1.00	−1.12	−1.03
PEX19	peroxisomal biogenesis factor 19	19298	1.61	2.25	1.19	1.36**	1.12	1.15	1.32**
PEX26	peroxisomal biogenesis factor 26	74043	−1.32	−1.86	1.01	1.26	1.01	1.29	1.10
PEX3	peroxisomal biogenesis factor 3	56535	1.50	1.77	1.13	1.37**	−1.05	1.09	1.20*
PEX6	peroxisomal biogenesis factor 6	224824	1.08	−1.06	1.12	1.16	1.30	−1.08	1.09
PXMP2	peroxisomal membrane protein 2	19301	−1.22	−1.29	−1.08	−1.20*	−1.28	−1.13	−1.06
PXMP4	peroxisomal membrane protein 4	59038	1.62	2.09	1.61	1.62*	1.99	−1.03	1.01

[1] From Rosen et al. [1],
*Significantly different than control ($P \leq .03$),
**Significantly different than control ($P \leq .0025$).

Table 6: Average fold change for genes related to the inflammatory response in wild-type and PPARα-null male mice following a seven-day exposure to Wy-14,643[1], PFOA[1], or PFOS.

Symbol	Gene name	Entrez no.	WT				Null		
			Wy14,643 50 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg
APCS	amyloid P component, serum	20219	−1.50	−2.33	−1.23	−1.28	−1.19	1.41	1.13
C1QA	complement component 1QA	12259	−1.75	−1.40	−1.13	−1.17	−1.31	−1.24	−1.34**
C1R	complement component 1r	50909	−2.67	−1.78	−1.15	−1.23*	−1.22	1.16	−1.17*
C1S	complement component 1s	317677	−3.73	−2.53	−1.14	−1.62**	−1.52	1.06	−1.11
C2	complement component 2	12263	−2.56	−1.91	−1.37	−1.32*	−1.18	1.10	1.11
C3	complement component 3	12266	−1.41	−1.41	−1.04	−1.04	−1.22	1.13	1.08*
C4B	complement component 4B	12268	−2.35	−2.15	−1.08	−1.28	−1.91	1.15	−1.13
C4BP	complement component 4 binding prot	12269	−1.86	−1.82	−1.11	−1.19	1.02	1.39	1.13
C6	complement component 6	12274	−2.66	−1.27	−1.35	−1.08	1.90	1.12	1.06
C8A	complement component 8, alpha	230558	−3.62	−1.94	−1.17	−1.31*	−1.17	1.19	1.04
C8B	complement component 8, beta	110382	−5.25	−2.99	−1.20	−1.60**	−1.12	1.11	1.02
C8G	complement component 8, gamma	69379	−1.59	−1.35	−1.05	−1.17*	−1.34	−1.10	−1.17**
C9	complement component 9	12279	−2.12	−2.64	−1.35	−1.58**	−1.46	1.08	−1.19*
CFB	complement factor B	14962	−1.81	−1.77	−1.07	−1.26	−1.39	1.07	−1.11
CFH	complement factor H	12628	−2.39	−2.30	−1.19	−1.62	−1.76	1.45	−1.35
CFI	complement factor I	12630	−1.63	−1.77	−1.06	−1.15	−1.06	1.12	1.04
CRP	C−reactive protein	12944	−1.33	−1.39	−1.01	−1.15*	1.32	1.14	1.13
CTSC	cathepsin C	13032	−1.56	−2.52	1.01	−1.36	−1.96	1.04	−1.35
F10	coagulation factor X	14058	−1.62	−1.42	−1.09	−1.13	−1.00	1.07	−1.07
F11	coagulation factor XI	109821	−2.17	−2.68	−1.41	−2.08**	−1.08	−1.08	−1.34*
F12	coagulation factor XII	58992	−1.22	−1.35	−1.05	−1.14	−1.21	−1.07	−1.12*
F13B	coagulation factor XIII, B polypeptide	14060	−1.41	−1.54	−1.11	−1.22**	1.02	1.02	−1.12
F2	coagulation factor II (thrombin)	14061	−1.19	−1.20	−1.02	−1.13*	−1.10	1.02	−1.02
F5	coagulation factor V	14067	−1.78	−1.53	−1.09	−1.44*	−1.41	1.08	−1.34*
F7	coagulation factor VII	14068	−2.68	−2.15	−1.09	−1.46**	−1.23	1.03	−1.03
F9	coagulation factor IX	14071	−1.42	−1.43	−1.02	−1.39*	−1.33	1.07	−1.19
FGA	fibrinogen alpha chain	14161	−1.27	−1.75	1.00	−1.12	−1.07	1.05	−1.07
FGB	fibrinogen beta chain	110135	−1.32	−1.97	1.03	−1.15	−1.25	1.08	−1.07

Table 6: Continued.

Symbol	Gene name	Entrez no.	Wy14,643 50 mg/kg	PFOA 3 mg/kg	WT PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	Null PFOS 3 mg/kg	PFOS 10 mg/kg
FGG	fibrinogen gamma chain	99571	−1.14	−1.68	1.02	−1.15*	−1.08	1.04	−1.06
KLKB1	kallikrein B, plasma (Fletcher factor) 1	16621	−1.58	−1.76	−1.09	−1.39*	−1.05	−1.03	−1.18*
LUM	lumican	17022	−1.34	−1.27	1.02	−1.20*	−1.66	1.03	−1.27
MASP1	Mannan-binding lectin1	17174	−1.23	−1.62	−1.19	−1.18*	1.11	1.18	1.17*
MBL2	Mannose-binding lectin 2	17195	−1.77	−2.18	−1.12	−1.23*	−1.36	−1.20	−1.28**
ORM2	orosomucoid 2	18405	−1.96	−2.04	−1.26	−1.21	−1.16	1.30	1.05
PROC	protein C	19123	−1.49	−1.50	−1.02	−1.13*	−1.09	−1.01	−1.09*
SAA1	serum amyloid A1	20209	−3.71	−3.98	−2.75	1.04	−2.76	6.51	2.55
SAA2	serum amyloid A2	20210	−1.75	−1.30	−1.79	−1.29	3.05	1.44	1.22
SAA4	serum amyloid A4, constitutive	20211	−2.19	−1.45	−1.06	−1.27	−1.02	1.47	−1.05
SERPINA1	serpin peptidase inhibitor, clade A1	20701	−3.43	−2.07	−1.03	−1.05**	−1.16	1.11	−1.33
SERPINC1	serpin peptidase inhibitor, clade C1	11905	−1.19	−1.21	−1.03	−1.08*	−1.02	−1.04	−1.06*
SERPIND1	serpin peptidase inhibitor, clade D1	15160	−1.62	−1.70	−1.08	−1.25**	−1.05	1.09	1.05
SERPINE1	serpin peptidase inhibitor, clade E1	18787	1.44	9.75	1.03	1.85**	2.95	1.03	1.26*
SERPINF2	serpin peptidase inhibitor, clade F2	18816	−1.15	−1.87	1.01	−1.13*	1.02	1.12	1.05
SERPING1	serpin peptidase inhibitor, clade G1	12258	−1.23	−1.37	−1.12	−1.13	−1.07	1.12	1.02
VWF	von Willebrand factor	22371	1.06	1.12	−1.25	1.07	−1.51	1.22	1.14

[1] From Rosen et al. [1],
*Significantly different than control ($P \leq .03$),
**Significantly different than control ($P \leq .0025$).

were included in each dose group. Dose levels were based on unpublished data from our laboratory and reflect exposures that produce hepatomegaly in adult mice without inducing overt toxicity. Animals utilized for RT-PCR analysis were taken from a separate set of WT and Null mice. PCR dose groups consisted of 4 animals per group and were treated for seven-days with either 10 mg/kg/day PFOS, 3 mg/kg/day PFOA (ammonium salt, catalog no. 77262, Sigma-Aldrich) in 0.5% Tween 20, or 50 mg/kg/day Wy-14,643 (catalog no. C7081, Sigma-Aldrich) in 0.5% methylcellulose, along with vehicle controls. All dosing solutions were freshly prepared each day. At the end of the dosing period, animals were euthanized by CO_2 asphyxiation and tissue collected from the left lobe of the liver for preparation of total RNA. Tissue prepared for histology was collected from the same group of animals used for microarray analysis and was taken from a section adjacent to that utilized for RNA preparation.

2.2. RNA Preparation. Collected tissue (≤50 mg) was immediately placed in 1 mL RNA*later* (Applied Biosystems/Ambion, Austin, TX) and stored at −20°C. RNA preparations for microarray analysis were then completed by homogenizing the tissue in 1 mL TRI reagent (Sigma Chemical) followed by processing through isopropanol precipitation according to the manufacturer's instructions. The resulting pellets were washed with 80% ethanol and resuspended in RNase free water (Applied Biosystems/Ambion). Preparations were further purified by passing approximately 100 μg per sample through RNeasy spin columns (Qiagen, Valencia, CA). RNA for PCR analysis was prepared using the *mir*VANA miRNA isolation kit (Applied Biosystems/Ambion) according to the manufacturer's protocol without further enrichment for small RNAs. All samples used in the study were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington,

Table 7: Average fold change for genes related to xenobiotic metabolism in wild-type and PPARα-null male mice following a seven-day exposure to Wy-14,643[1], PFOA[1], or PFOS.

					WT			Null	
Symbol	Gene name	Entrez no.	Wy14,643 50 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg
ADH1C	alcohol dehydrogenase 1C	11522	1.27	1.02	−1.00	1.02	−1.09	−1.02	−1.04
ADH5	alcohol dehydrogenase 5	11532	−1.18	1.10	1.09	−1.04	−1.02	1.11	1.14
ADH7	alcohol dehydrogenase 7	11529	−1.51	1.06	−1.01	−1.06	−1.71	−1.01	−1.01
ALDH1L1	aldehyde dehydrogenase 1L1	107747	−1.29	−1.85	−1.08	−1.18*	−1.41	1.76	1.68**
ALDH3B1	aldehyde dehydrogenase 3B1	67689	1.12	1.04	−1.11	1.04	1.48	−1.03	−1.11
CES1	carboxylesterase 1	12623	1.43	2.29	1.61	2.62**	3.15	4.80	4.84**
CES2	carboxylesterase 2	234671	3.37	5.75	1.03	2.29	4.25	1.41	1.74*
CYP1A1	cytochrome P450,1A1	13076	1.25	−1.93	−1.05	1.08	−1.02	1.34	1.49**
CYP1A2	cytochrome P450,1A2	13077	−1.67	−1.24	−1.13	1.10	1.26	1.15	1.25*
CYP2A4	cytochrome P450,2A4	13087	−4.26	1.33	1.08	2.01	5.82	1.28	1.57**
CYP2B10	cytochrome P450,2B10	13088	1.31	4.39	3.50	5.92*	24.20	11.34	21.66**
CYP2C55	cytochrome P450,2C55	72082	1.58	21.72	1.54	8.37*	110.35	10.57	25.18**
CYP2C37	cytochrome P450,2C37	13096	−2.42	1.57	1.39	1.48	4.09	1.53	1.68
CYP2C38	cytochrome P450, 2C38	13097	1.62	1.12	1.78	2.30**	−1.42	−1.26	1.03
CYP2C39	cytochrome P450, 2C39	13098	2.45	1.51	1.65	1.51	−1.42	1.11	−1.01
CYP2C50	cytochrome P450,2C50	107141	−2.63	1.31	1.11	1.19	1.71	1.34	1.26
CYP2C54	cytochrome P450,2C54	404195	−2.98	1.44	1.16	1.14	1.87	1.29	1.35**
CYP2C70	cytochrome P450,2C70	226105	−2.75	−4.22	−1.23	−1.68*	−1.05	−1.05	1.04
CYP2C65	cytochrome P450,2C65	72303	1.44	1.63	−1.93	1.98	46.78	2.28	8.63**
CYP2D10	cytochrome P450,2D10	13101	−1.47	−1.09	−1.02	−1.03	1.33	−1.00	1.02
CYP2D26	cytochrome P450,2D26	76279	−1.17	−1.21	1.06	−1.01	−1.12	−1.03	−1.08
CYP3A11	cytochrome P450,3A11	13112	−1.23	1.40	1.03	1.06	4.61	1.12	1.20
CYP3A41A	cytochrome P450,3A41A	53973	−2.08	1.11	1.24	1.58*	2.01	1.39	1.25
CYP3A25	cytochrome P450,3A25	56388	−1.94	−1.70	1.01	−1.01	1.04	1.13	1.12
CYP3A13	cytochrome P450,3A13	13113	−1.54	1.19	1.22	1.38*	1.52	1.75	1.62**
EPHX1	epoxide hydrolase 1, microsomal	13849	1.22	1.78	1.16	1.60*	1.82	1.33	1.59*
EPHX2	epoxide hydrolase 2, cytoplasmic	13850	2.25	2.34	1.45	1.67**	1.04	1.05	1.07

Table 7: Continued.

Symbol	Gene name	Entrez no.	Wy14,643 50 mg/kg	PFOA 3 mg/kg	WT PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	Null PFOS 3 mg/kg	PFOS 10 mg/kg
GSTA3	glutathione S-transferase A3	14859	1.08	−1.04	1.05	1.26	1.11	1.11	1.13
GSTA4	glutathione S-transferase A4	14860	−2.01	−1.10	−1.02	1.52	1.37	−1.20	1.36
GSTA5	glutathione S-transferase A5	14857	−1.12	1.44	1.19	2.76*	2.26	1.15	2.13
GSTK1	glutathione S-transferase kappa 1	76263	1.85	1.43	1.02	−1.04	−1.30	−1.26	−1.27
GSTM1	glutathione S-transferase M1	14863	−2.12	−1.56	−1.51	1.77	2.54	1.18	1.97
GSTM3	glutathione S-transferase, mu 3	14864	−1.32	1.50	1.16	2.44*	1.83	1.57	2.59*
GSTM4	glutathione S-transferase M4	14865	2.07	3.13	1.30	2.40*	2.48	1.40	2.63*
GSTP1	glutathione S-transferase pi 1	14870	−2.79	4.14	−1.16	1.00	2.87	−1.06	−1.03
GSTT2	glutathione S-transferase theta 2	14872	1.64	2.74	1.42	1.83**	1.13	1.16	1.43**
GSTT3	glutathione S-transferase, theta 3	103140	2.10	1.13	1.41	1.61	1.77	1.30	1.85**
GSTZ1	glutathione transferase zeta 1	14874	−1.36	−1.14	−1.03	−1.08	1.01	1.03	1.01
MGST1	microsomal glutathione S-transferase 1	56615	1.28	1.24	−1.02	1.01	1.21	1.04	1.01
MGST3	microsomal glutathione S-transferase 3	66447	1.73	1.60	1.24	1.80*	−1.54	−1.31	−1.06
POR	P450 (cytochrome) oxidoreductase	18984	−1.26	2.63	1.27	1.94	2.04	2.91	3.30**
UGT2B17	UDP glucuronosyltransferase 2B17	71773	−3.90	−1.13	−1.03	1.02	1.24	1.03	−1.01
UGT2B4	UDP glucuronosyltransferase 2B4	552899	−1.37	−1.93	−1.26	−1.23*	1.35	1.01	1.03
UGT2B7	UDP glucuronosyltransferase 2B7	231396	−1.19	−1.20	−1.05	−1.05	1.16	1.04	−1.00

[1] From Rosen et al. (2008),
*Significantly different than control ($P \leq .03$),
**Significantly different than control ($P \leq .0025$).

DE) and quality evaluated using a 2100 Bioanalyzer (Agilent, Palo Alto, CA). Only samples with an RNA Integrity number of at least 8.0 (2100 Expert software, version B.01.03) were included in the study [35].

2.3. Histological Examination of Tissue. Following overnight fixation in Bouins fixative, collected tissue was washed three times in PBS, dehydrated to 70% ethanol, and stored at 4°C until use. On the day of embedding, the tissue was dehydrated through an ethanol gradient to 100% ethanol and paraffin embedded using standard techniques. Five micron sections were then prepared using a rotary microtome prior to routine staining with hematoxylin and eosin.

2.4. Gene Profiling. Microarray analysis was conducted at the U.S. EPA NHEERL Toxicogenomics Core Facility using Affymetrix GeneChip 430_2 mouse genome arrays according to the protocols recommended by the manufacturer (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was produced from 5 ug total RNA using Enzo Single-Round RNA Amplification and Biotin Labeling System (Cat. no. 42420-10, Enzo Life Sciences Inc, Farmingdale, NY), quantified

Table 8: Average fold change for genes related to cholesterol biosynthesis in wild-type and PPARα-null male mice following a seven-day exposure to Wy-14,643[1], PFOA[1], or PFOS.

Symbol	Gene name	Entrez no.	WT Wy14,643 50 mg/kg	WT PFOA 3 mg/kg	WT PFOS 3 mg/kg	WT PFOS 10 mg/kg	Null PFOA 3 mg/kg	Null PFOS 3 mg/kg	Null PFOS 10 mg/kg
CYP51	cytochrome P450, family 51	13121	2.85	1.37	1.27	2.10*	1.37	2.99	1.93**
FDFT1	farnesyl-diphosphate farnesyltransferase 1	14137	2.30	1.28	1.29	1.73*	1.09	2.00	1.92**
FDPS	farnesyl diphosphate synthase	110196	3.19	1.79	1.16	1.38	1.83	1.84	1.96**
HMGCR	3-hydroxy-3-methylglutaryl -CoA reductase	15357	1.79	−1.08	1.19	1.97**	1.20	1.85	1.80*
HMGCS1	3-hydroxy-3-methylglutaryl -CoA synthase 1	208715	6.67	1.79	1.15	1.61	−1.06	3.11	1.86*
HMGCS2	3-hydroxy-3-methylglutaryl -CoA synthase 2	15360	1.17	1.54	1.28	1.34*	1.25	−1.08	−1.28*
IDI1	isopentenyl-diphosphate delta isomerase 1	319554	3.14	1.61	1.35	1.62	1.40	1.96	1.57*
LSS	lanosterol synthase	16987	1.73	1.08	1.12	1.41	−1.26	1.98	2.13**
MVK	mevalonate kinase	17855	1.45	−1.24	1.12	1.22	−1.02	1.57	1.52**
PMVK	phosphomevalonate kinase	68603	3.23	2.04	1.36	1.51*	1.20	1.58	1.53**
SQLE	squalene epoxidase	20775	3.10	1.05	1.17	1.46	1.26	2.25	1.98**

[1] From Rosen et al. (2008), *Significantly different than control ($P \leq .03$), **Significantly different than control ($P \leq .0025$).

using an ND-1000 spectrophotometer, and evaluated on a 2100 Bioanalyzer after fragmentation. To minimize technical day to day variation, labeling and hybridization for all samples were conducted as a single block. Following overnight hybridization at 45°C in an Affymetrix Model 640 GeneChip hybridization oven, the arrays were washed and stained using an Affymetrix 450 fluidics station and scanned on an Affymetrix Model 3000 scanner. Raw data (Affymetrix Cel files) were obtained using Affymetrix GeneChip Operating Software (version 1.4). This software also provided summary reports by which array QA metrics were evaluated including average background, average signal, and 3′/5′ expression ratios for spike-in controls, β-actin, and GAPDH. Only arrays of high quality based on low background levels as well as expected 3′/5′ expression ratios for the spike-in controls, β-actin, and GAPDH were included in the study. Data are available through the Gene Expression Omnibus at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo) as accession numbers GSE22871.

2.5. PCR Confirmation of Results. Real-time PCR analysis of selected genes was conducted using 2 micrograms of total RNA. All samples were initially digested using 2 units DNaseI (no. M6101, Promega Corporation, Madison, WI) for 30 min at 37°C followed by 10 min at 65°C in a buffer containing 40 mM Tris (pH 8.0), 10 mM $MgSO_4$, and 1 mM $CaCl_2$. The RNA was then quantified using a Quant-iT RiboGreen RNA assay kit according to the manufacturer's protocol (no.R11490, Invitrogen Corporation, Carlsbad, CA) and approximately 1.5 ug RNA reverse transcribed using a High Capacity cDNA Archive Kit according to the provided protocol (no. 4322171, Applied Biosystems, Foster City, CA). Amplification was performed on an Applied Biosystems model 7900HT Fast Real-Time PCR System in duplicate using 25 ng cDNA and TaqMan Universal PCR Master Mix (no.4304437, Applied Biosystems) in a total volume of 12 μL according to the protocol supplied by the manufacturer. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Entrez no. 14433), which was uniformly expressed among all samples (cycle threshold deviation less than 0.35), was used as an endogenous reference gene. The following TaqMan assays (Applied Biosystems) were included in the study: *Gapdh* (no. Mm99999915_g1), *Srebf2* (no. Mm01306293_m1), *Ppargc1a* (Mm0047183_m1), *Nfe2l2 (Mm00477784_m1)*, *Ndufa5* (Mm00471676), *Lss* (no. Mm00461312_m1), *Cyp4a14* (no. Mm00484132_m1), *Cyp7a1* (no. Mm00484152_m1), and *Cyp2b10* (no. Mm00456591_m1). Fold change was calculated using the $2^{-\Delta\Delta C}$T method of Livak and Schmittgen [36].

2.6. Data Analysis. Body and liver weight data were analyzed by strain using a one-way ANOVA. Individual treatment contrasts were assessed using a Tukey Kramer HSD test ($P \leq .05$) (JMP 7.0 (SAS, Cary, NC). Microarray data were summarized, background adjusted, and quantile

Table 9: Average fold change for genes related to oxidative phosphorylation/electron transport in wild-type and PPARα-null male mice following a seven-day exposure to Wy-14,643[1], PFOA[1], or PFOS.

			WT				Null		
Symbol	Gene name	Entrez no.	Wy14,643 50 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg
ATP5D	ATP synthase H+ transporting, F1delta	66043	1.03	1.10	1.04	1.09	−1.17	−1.22	−1.13*
ATP5E	ATP synthase H+ transporting, F1epsilon	67126	−1.10	1.21	−1.00	1.03	−1.17	−1.32	−1.38**
ATP5G2	ATP synthase H+ transporting, F0, C2	67942	−1.09	−1.03	1.10	−1.10	−1.10	−1.33	−1.26**
ATP5G3	ATP synthase H+ transporting, F0, C3	228033	1.62	1.48	−1.01	1.05	−1.10	−1.12	−1.10**
ATP5H	ATP synthase H+ transporting, F0, D	71679	1.18	1.10	1.05	1.06	−1.01	−1.30	−1.38**
ATP5I	ATP synthase H+ transporting, F0, E	11958	−1.01	−1.45	−1.03	1.10	1.17	−1.38	−1.50**
ATP5J	ATP synthase H+ transporting, F0, F6	11957	−1.20	1.44	−1.04	−1.07	−1.14	−1.25	−1.35**
ATP5J2	ATP synthase H+ transporting,F0, F2	57423	2.38	−1.56	−1.05	−1.09	1.03	−1.29	−1.35**
ATP5L	ATP synthase H+ transporting, F0, G	27425	1.58	1.21	−1.02	1.00	−1.05	−1.33	−1.30**
ATP5O	ATP synthase H+ transporting, F1, O	28080	1.12	1.16	1.06	1.22	−1.03	−1.33	−1.31**
ATP6V0B	ATPase, H+ transporting, V0 unit b	114143	−1.37	−1.25	1.03	−1.09	1.05	−1.22	−1.20**
ATP6V1F	ATPase, H+ transporting, V1 unit F	66144	−1.18	1.23	1.00	1.05	1.01	−1.33	−1.28**
COX4I1	cytochrome c oxidase unit IV isoform 1	12857	1.14	1.15	1.02	1.03	−1.15	−1.19	−1.16**
COX5A	cytochrome c oxidase unit Va	12858	1.25	1.12	−1.02	1.09	−1.13	−1.26	−1.33**
COX5B	cytochrome c oxidase unit Vb	12859	1.19	1.33	1.09	1.08	−1.27	−1.27	−1.35**
COX6B1	cytochrome c oxidase unit VIb1	110323	1.32	1.39	−1.01	1.10*	−1.12	−1.25	−1.19*
COX6C	cytochrome c oxidase unit VIc	12864	1.62	−1.23	1.03	−1.05	1.21	−1.22	−1.25**
COX7A2	cytochrome c oxidase unit VIIa 2	12866	−1.68	−1.08	−1.04	−1.04	−1.57	−1.39	−1.37**
COX7C	cytochrome c oxidase unit VIIc	12867	1.22	1.32	−1.03	−1.28*	−1.05	−1.23	−1.19**
COX8A	cytochrome c oxidase unit 8A	12868	1.34	1.34	1.02	1.04	1.07	−1.23	−1.13*

Table 9: Continued.

Symbol	Gene name	Entrez no.	WT Wy14,643 50 mg/kg	WT PFOA 3 mg/kg	WT PFOS 3 mg/kg	WT PFOS 10 mg/kg	Null PFOA 3 mg/kg	Null PFOS 3 mg/kg	Null PFOS 10 mg/kg
NDUFA1	NADH dehydrogenase 1 alpha1	54405	−1.19	1.13	−1.03	−1.11	−1.25	−1.31	−1.49**
NDUFA2	NADH dehydrogenase 1 alpha 2	17991	1.06	1.18	1.04	1.04	−1.06	−1.26	−1.33**
NDUFA3	NADH dehydrogenase 1 alpha 3	66091	1.60	1.60	1.06	1.16*	−1.06	−1.37	−1.30**
NDUFA4	NADH dehydrogenase 1 alpha 4	17992	1.02	2.46	−1.00	1.01	3.16	−1.12	−1.11**
NDUFA5	NADH dehydrogenase 1 alpha 5	68202	1.41	1.26	1.10	1.11	−1.07	−1.55	−1.73**
NDUFA6	NADH dehydrogenase 1 alpha 6	67130	1.10	1.06	1.02	−1.04	−1.02	−1.34	−1.29**
NDUFA7	NADH dehydrogenase 1 alpha 7	66416	−1.14	−1.01	1.09	1.12	−1.17	−1.45	−1.38**
NDUFA8	NADH dehydrogenase 1 alpha 8	68375	1.14	1.33	1.00	1.09	1.05	−1.29	−1.18*
NDUFA12	NADH dehydrogenase 1 alpha12	66414	1.47	1.16	−1.03	1.06	1.06	−1.51	−1.40**
NDUFA13	NADH dehydrogenase 1 alpha13	67184	−1.12	−1.16	−1.03	−1.03	−1.08	−1.26	−1.28**
NDUFA9	NADH dehydrogenase 1 alpha 9	66108	1.18	1.07	1.02	−1.01	−1.09	−1.20	−1.19**
NDUFAB1	NADH dehydrogenase 1, alpha/beta 1	70316	1.56	1.19	1.05	1.23*	−1.07	−1.31	−1.44*
NDUFB2	NADH dehydrogenase 1 beta 2	68198	−2.31	−3.32	1.04	1.11	1.49	−1.31	−1.35**
NDUFB3	NADH dehydrogenase 1 beta 3	66495	1.55	1.93	1.09	1.19	1.05	−1.41	−1.32**
NDUFB4	NADH dehydrogenase 1 beta 4	68194	−1.03	1.17	−1.01	1.06	−1.13	−1.45	−1.46**
NDUFB5	NADH dehydrogenase 1 beta 5	66046	1.21	1.13	1.08	1.03	1.05	−1.28	−1.41**
NDUFB6	NADH dehydrogenase 1 beta 6,	230075	1.32	−1.03	1.04	1.19	−1.02	−1.38	−1.36**
NDUFB7	NADH dehydrogenase 1 beta 7,	66916	1.02	1.14	1.04	1.11	−1.11	−1.40	−1.29**
NDUFB9	NADH dehydrogenase 1 beta 9,	66218	1.19	1.01	1.05	1.01	−1.08	−1.22	−1.25**
NDUFB11	NADH dehydrogenase 1 beta 11	104130	−1.29	1.05	1.05	1.06	−1.00	−1.26	−1.23**
NDUFC1	NADH dehydrogenase 1 unknown 1	66377	−1.28	1.84	1.07	1.21*	1.17	−1.28	−1.37**
NDUFC2	NADH dehydrogenase 1 unknown, 2	68197	−1.02	1.13	1.06	1.06	−1.13	−1.37	−1.33**
NDUFS4	NADH dehydrogenase Fe-S protein 4	17993	1.51	1.21	1.12	−1.12	1.07	−1.41	−1.40**
NDUFS5	NADH dehydrogenase Fe-S protein 5	595136	1.16	1.13	−1.01	1.08	1.02	−1.37	−1.44**
NDUFS7	NADH dehydrogenase Fe-S protein 7	75406	1.09	1.40	1.09	1.13*	1.07	−1.28	−1.15

Table 9: Continued.

Symbol	Gene name	Entrez no.	Wy14,643 50 mg/kg	PFOA 3 mg/kg	WT PFOS 3 mg/kg	WT PFOS 10 mg/kg	Null PFOA 3 mg/kg	Null PFOS 3 mg/kg	Null PFOS 10 mg/kg
NDUFS6	NADH dehydrogenase Fe-S protein 6	407785	−1.32	1.06	−1.01	1.02	−1.14	−1.30	−1.32**
NDUFV2	NADH dehydrogenase flavoprotein 2	72900	1.38	1.09	1.06	1.07	−1.02	−1.24	−1.24**
NDUFV3	NADH dehydrogenase flavoprotein 3,	78330	1.12	1.16	−1.03	−1.01	−1.14	−1.35	−1.39**
UCRC	ubiquinol-cytochrome c reductase	66152	1.58	1.26	1.10	1.27	1.07	−1.40	−1.27**
UHRF1BP1	UHRF1 binding protein 1	224648	−1.03	1.36	−1.08	1.06	1.15	1.23	1.15**
UQCR	ubiquinol-cytochrome c reductase	66594	1.26	1.40	1.04	1.14*	1.09	−1.28	−1.19*
UQCRC2	ubiquinol-cytochrome c reductase CP II	67003	1.09	1.17	1.07	1.13	−1.04	−1.11	−1.27*
UQCRQ	ubiquinol-cytochrome c reductase 3 unit 7	22272	1.01	1.08	1.07	1.12*	−1.07	−1.18	−1.21**

[1] From Rosen et al. [1], *Significantly different than control ($P \leq .03$),**Significantly different than control ($P \leq .0025$).

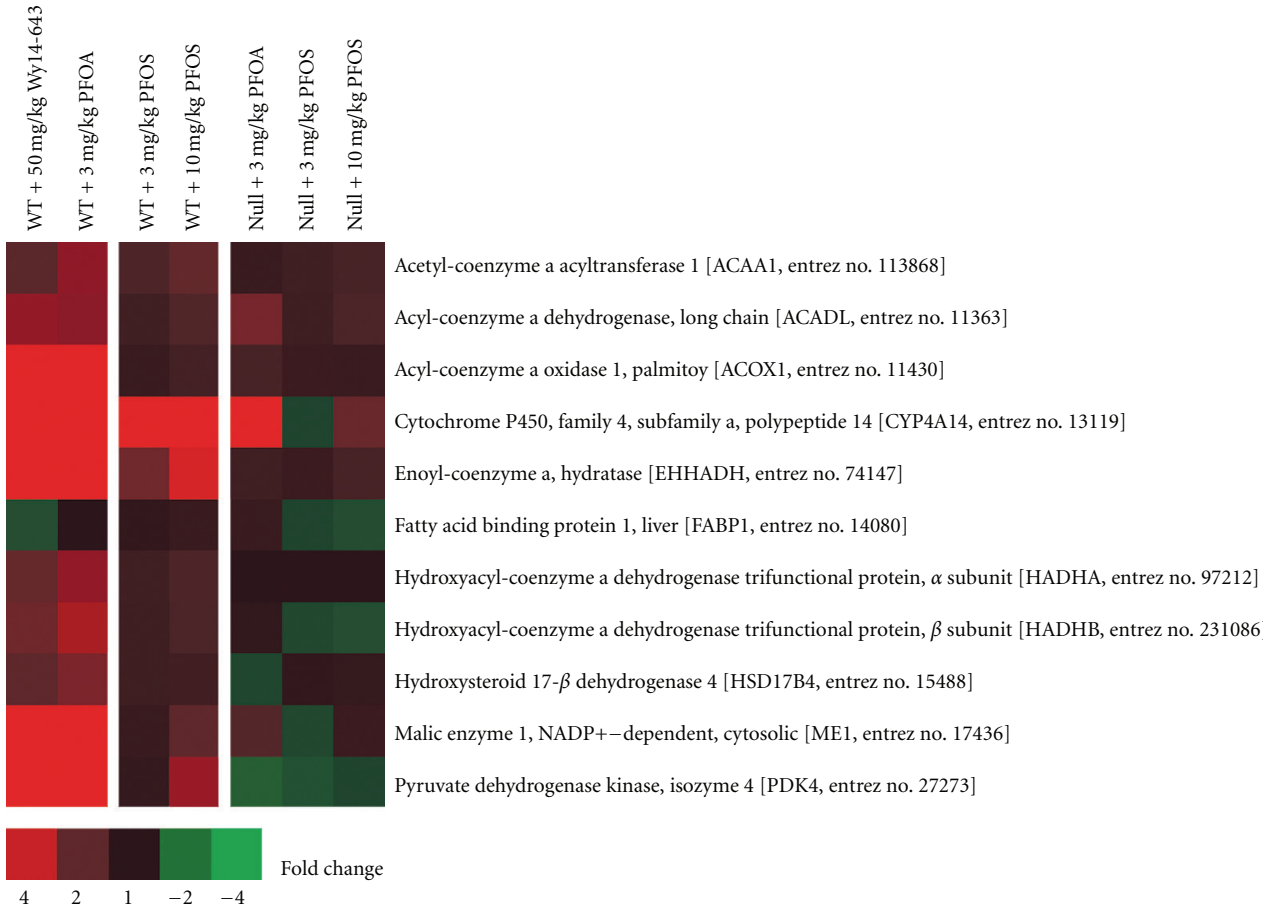

Figure 2: Expression of a group of well characterized markers of PPARα transactivation in WT and Null mice. The response to PFOS in WT mice was less robust than that previously observed for either PFOA or Wy14,643. Red or green correspond to average up- or down-regulation, respectively.

Table 10: Average fold change for genes related to ribosome biogenesis following a seven-day exposure to Wy-14,643[1], PFOA[1], or PFOS in wild-type and PPARα-null male mice.

Symbol	Gene name	Entrez no.	WT				Null		
			Wy14,643 50 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg
MRPL12	mitochondrial ribosomal protein L12	56282	−1.16	1.25	1.07	1.14*	−1.16	−1.18	−1.12*
MRPL13	mitochondrial ribosomal protein L13	68537	1.32	1.33	1.12	1.35*	1.01	−1.21	−1.42**
MRPL17	mitochondrial ribosomal protein L17	27397	1.68	1.76	1.10	1.43**	1.13	−1.13	1.09
MRPL23	mitochondrial ribosomal protein L23	19935	−1.14	−1.04	−1.00	1.10	1.09	−1.38	−1.20*
MRPL33	mitochondrial ribosomal protein L33	66845	1.22	1.26	1.07	1.05	1.04	−1.29	−1.28**
MRPS12	mitochondrial ribosomal protein S12	24030	−1.24	1.18	1.05	1.12	1.02	−1.27	−1.15
MRPS18A	mitochondrial ribosomal protein S18A	68565	−1.46	1.34	1.04	1.28*	1.60	−1.19	−1.06
RPL10	ribosomal protein L10	110954	−1.15	−1.21	1.02	1.03	1.07	−1.10	−1.02
RPL10A	ribosomal protein L10A	19896	−1.11	1.10	1.03	1.05	1.00	−1.07	1.01
RPL11	ribosomal protein L11	67025	1.14	1.12	1.10	1.11*	1.15	−1.15	−1.09
RPL12	ribosomal protein L12	269261	1.01	1.37	1.08	1.15*	1.11	−1.08	1.05
RPL13A	ribosomal protein L13a	22121	−1.14	1.03	1.07	1.12*	−1.17	−1.15	−1.10
RPL14	ribosomal protein L14	67115	−1.28	−1.06	1.15	1.23**	−1.13	−1.18	−1.22*
RPL17	ribosomal protein L17	319195	−1.27	1.15	1.03	1.12	−1.52	−1.10	−1.09
RPL18	ribosomal protein L18	19899	−1.11	1.28	1.04	1.07*	1.19	−1.27	−1.09*
RPL18A	ribosomal protein L18a	76808	1.65	−1.37	1.04	1.11*	1.08	−1.15	−1.02
RPL19	ribosomal protein L19	19921	1.22	1.23	1.01	1.05	1.07	−1.11	−1.03
RPL21	ribosomal protein L21	19933	2.00	1.55	1.03	1.09	1.18	−1.20	−1.18
RPL22	ribosomal protein L22	19934	1.17	1.45	1.06	1.29**	1.08	−1.25	−1.14*
RPL23	ribosomal protein L23	65019	−1.07	1.35	1.06	1.06	1.22	−1.24	−1.16
RPL24	ribosomal protein L24	68193	−1.13	1.07	1.06	1.09*	−1.00	−1.19	−1.11*
RPL26	ribosomal protein L26	19941	1.04	1.22	1.03	1.03	1.07	−1.22	−1.18**
RPL27	ribosomal protein L27	19942	1.04	−1.01	1.08	1.38**	1.06	−1.25	−1.40*
RPL27A	ribosomal protein L27a	26451	−1.07	1.07	−1.00	1.17	1.26	−1.17	−1.09
RPL28	ribosomal protein L28	19943	1.29	1.04	1.01	1.11*	1.67	−1.22	−1.10
RPL29	ribosomal protein L29	19944	1.16	−1.30	1.04	1.09	1.08	−1.23	−1.17

Table 10: Continued.

Symbol	Gene name	Entrez no.	WT				Null		
			Wy14,643 50 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg	PFOA 3 mg/kg	PFOS 3 mg/kg	PFOS 10 mg/kg
RPL3	ribosomal protein L3	27367	−1.00	−1.14	1.01	1.09	−1.01	−1.03	1.06
RPL30	ribosomal protein L30	19946	−1.15	−1.07	1.02	−1.21	−1.04	−1.29	−1.23**
RPL31	ribosomal protein L31	114641	1.11	1.37	1.09	1.05	1.29	−1.18	−1.12*
RPL32	ribosomal protein L32	19951	1.06	1.11	1.02	1.12*	1.08	−1.16	−1.03
RPL34	ribosomal protein L34	68436	−1.26	1.16	−1.07	1.05	−1.04	−1.22	−1.31**
RPL35	ribosomal protein L35	66489	−1.03	1.15	1.13	1.26**	1.04	−1.17	−1.11
RPL36	ribosomal protein L36	54217	−1.07	1.12	1.09	1.23*	1.07	−1.27	−1.20*
RPL37	ribosomal protein L37	67281	−1.16	−1.18	1.04	1.27*	1.17	−1.19	−1.10**
RPL37A	ribosomal protein L37a	19981	−1.15	−1.09	1.03	1.16	−1.12	−1.22	−1.19*
RPL38	ribosomal protein L38	67671	−1.17	1.14	−1.01	1.06	−1.03	−1.18	−1.10
RPL39	ribosomal protein L39	67248	1.04	1.02	1.06	1.13*	1.07	−1.18	−1.16**
RPL4	ribosomal protein L4	67891	1.16	1.43	1.03	1.03	1.32	1.03	1.04
RPL41	ribosomal protein L41	67945	−1.06	1.14	1.05	1.06	−1.13	−1.20	−1.26*
RPL5	ribosomal protein L5	19983	−1.21	1.02	1.24	1.09*	−1.05	−1.05	−1.11
RPL6	ribosomal protein L6	19988	1.01	−1.08	1.00	1.05	1.15	−1.05	1.03
RPL7A	ribosomal protein L7a	27176	−1.02	−1.11	1.01	1.01	−1.02	−1.07	1.01
RPL9	ribosomal protein L9	20005	−1.35	−1.08	1.03	1.07	−1.11	−1.19	−1.12*
RPS10	ribosomal protein S10	67097	−1.02	1.02	1.05	1.07	1.00	−1.17	−1.12*
RPS11	ribosomal protein S11	27207	1.05	−1.74	−1.01	1.11	1.06	−1.24	−1.14*
RPS12	ribosomal protein S12	20042	1.16	1.22	1.11	1.19	1.22	−1.21	−1.12
RPS13	ribosomal protein S13	68052	−1.03	1.10	1.07	1.22*	1.11	−1.27	−1.22*
RPS14	ribosomal protein S14	20044	−1.03	1.19	1.05	1.11*	1.01	−1.17	−1.11**
RPS15A	ribosomal protein S15a	267019	−1.05	1.05	1.02	1.12	1.02	−1.14	−1.20
RPS16	ribosomal protein S16	20055	−1.09	1.05	1.05	1.07	−1.02	−1.12	−1.07
RPS17	ribosomal protein S17	20068	1.00	1.16	1.04	−1.19*	1.01	−1.19	−1.15*
RPS19	ribosomal protein S19	20085	−1.07	1.23	1.08	1.19**	−1.00	−1.14	−1.05
RPS2	ribosomal protein S2	16898	−1.09	1.02	1.04	1.02	−1.16	−1.03	1.04

Table 10: Continued.

Symbol	Gene name	Entrez no.	WT Wy14,643 50 mg/kg	WT PFOA 3 mg/kg	WT PFOS 3 mg/kg	WT PFOS 10 mg/kg	Null PFOA 3 mg/kg	Null PFOS 3 mg/kg	Null PFOS 10 mg/kg
RPS20	ribosomal protein S20	67427	−1.40	1.21	1.04	1.15	1.25	−1.11	−1.13
RPS21	ribosomal protein S21	66481	1.11	−1.32	1.15	1.38	1.39	−1.32	−1.25**
RPS23	ribosomal protein S23	66475	1.01	1.04	−1.00	1.04	1.09	−1.21	−1.10*
RPS24	ribosomal protein S24	20088	1.58	1.62	1.11	−1.29*	1.75	−1.16	−1.19**
RPS25	ribosomal protein S25	75617	−1.23	1.01	1.09	1.13*	−1.02	−1.30	−1.17*
RPS26	ribosomal protein S26	27370	1.32	1.30	1.04	1.16*	1.14	−1.20	−1.08
RPS27A	ribosomal protein S27a	78294	1.05	−1.05	−1.00	1.02	1.09	−1.08	−1.05
RPS27L	ribosomal protein S27-like	67941	1.72	1.28	1.07	1.14*	1.19	−1.18	−1.17*
RPS28	ribosomal protein S28	54127	−1.19	−1.03	1.03	1.06	−1.05	−1.28	−1.17*
RPS29	ribosomal protein S29	20090	−1.26	−1.05	−1.02	1.01	−1.03	−1.19	−1.20**
RPS3	ribosomal protein S3	27050	−1.04	1.29	1.03	1.20*	−2.88	−1.11	−1.06
RPS3A	ribosomal protein S3A	544977	−1.18	−1.07	1.02	−1.01	−1.05	−1.10	−1.03
RPS5	ribosomal protein S5	20103	−1.16	1.18	1.06	1.09*	−1.02	−1.13	−1.00
RPS6	ribosomal protein S6	20104	−1.20	−1.02	−1.20	1.06	−1.02	−1.14	−1.06*
RPS8	ribosomal protein S8	20116	1.19	−1.05	1.07	1.13*	1.04	−1.29	−1.13
RPS9	ribosomal protein S9	76846	−1.39	1.30	1.05	1.07	1.05	−1.08	−1.04

[1] From Rosen et al. (2008), *Significantly different than control ($P \leq .03$),
**Significantly different from control ($P \leq .0025$).

normalized using Robust Multichip Average methodology (RMA Express, ver. 1.0). Prior to statistical analysis, microarray data were filtered to remove probe sets with weak or no signal. Data were analyzed for each strain using a one-way ANOVA across dose (Proc GLM, SAS ver. 9.1, Cary, NC). Individual treatment contrasts were evaluated using a pairwise t-test of the least square means. Significant probe sets ($P \leq .0025$) were evaluated for relevance to biological pathway and function using Ingenuity Pathway Analysis software (http://analysis.ingenuity.com/) and DAVID functional annotation software [37]. Duplicate probe sets were resolved using minimum P-value. Data were further evaluated without statistical filtering using Gene Set Enrichment Analysis (GSEA) software available from the Broad Institute [38]. Hierarchical clustering and heat maps were generated using Eisen Lab Cluster and Treeview software (version 2.11).

3. Results

3.1. Necropsy and Histopathology. Liver weight increased at the highest dose of PFOS in both WT and Null animals (Table 1). Histological changes were also noted. Vacuole formation was observed in tissue sections from treated WT mice, as well as in sections from control and treated Null mice (Figure 1). The origin of these vacuoles was not fully apparent. Kudo and Kawashima [28] reported that chronic exposure to PFOA can induce fatty liver in mice due to altered triglyceride transport; hence, vacuolization in the current study may be the result of similar changes in WT mice. In Null mice, vacuole formation may also reflect increased triglyceride retention due to reduced hepatic fatty acid catabolism. Furthermore, our group has suggested that a certain degree of vacuolization may be unrelated to triglyceride retention in PFOA-exposed Null mice [29]. It

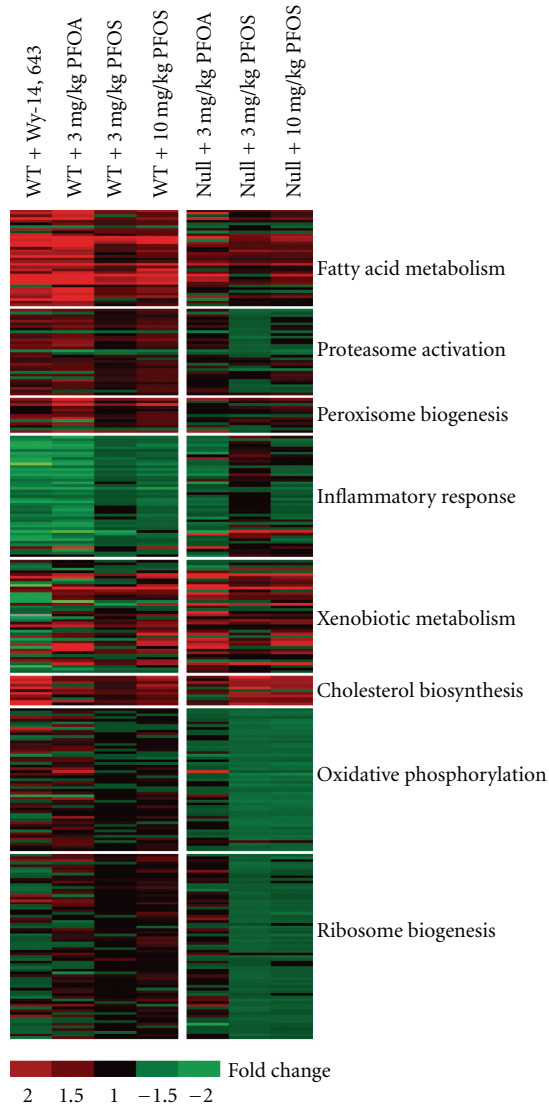

Figure 3: Functional categories of genes modified by PFOS in WT and Null mice. In WT mice, PFOS altered the expression of genes related to a variety of PPARα-regulated functions including lipid metabolism, peroxisome biogenesis, proteasome activation, and the inflammatory response. Genes affected in both WT and Null mice consisted of transcripts related to lipid metabolism, inflammation, and xenobiotic metabolism. Several categories of genes were uniquely regulated by PFOS in Null mice including up-regulation of genes in the cholesterol biosynthesis pathway as well as modest down-regulation of genes associated with oxidative phosphorylation and ribosome biogenesis. Red or green corresponds to average up- or down- regulation, respectively.

is possible therefore, that hepatic vacuolization might be associated with the liver weight increase observed in treated Null animals.

3.2. Gene Profiling. Based on the number of genes significantly altered by PFOS ($P \leq .0025$), gene expression changes in WT mice were more evident at the higher dose of PFOS compared to the lower dose. This was in contrast to changes observed in Null mice where the number of transcripts influenced by PFOS was similar across either dose group. Hence, certain PPARα-independent effects were found to be robust in Null mice even at the lowest dose of PFOS. This pattern of gene expression also varied from that previously observed by our group for PFOA where only moderate changes were found in Null mice compared to WT animals [1] (Table 2). By examining the expression of a small group of well characterized markers of PPARα transactivation, PFOS also appeared to be a less robust activator of murine PPARα than PFOA (Figure 2), a conclusion formerly reported by others [18, 39, 40].

In WT mice, PFOS modified the expression of genes related to a variety of PPARα-regulated functions including lipid metabolism, peroxisome biogenesis, proteasome activation, and the inflammatory response. Genes affected in both WT and Null mice consisted of transcripts related to lipid metabolism, inflammation, and xenobiotic metabolism, including the CAR inducible gene, *Cyp2b10*. It should be

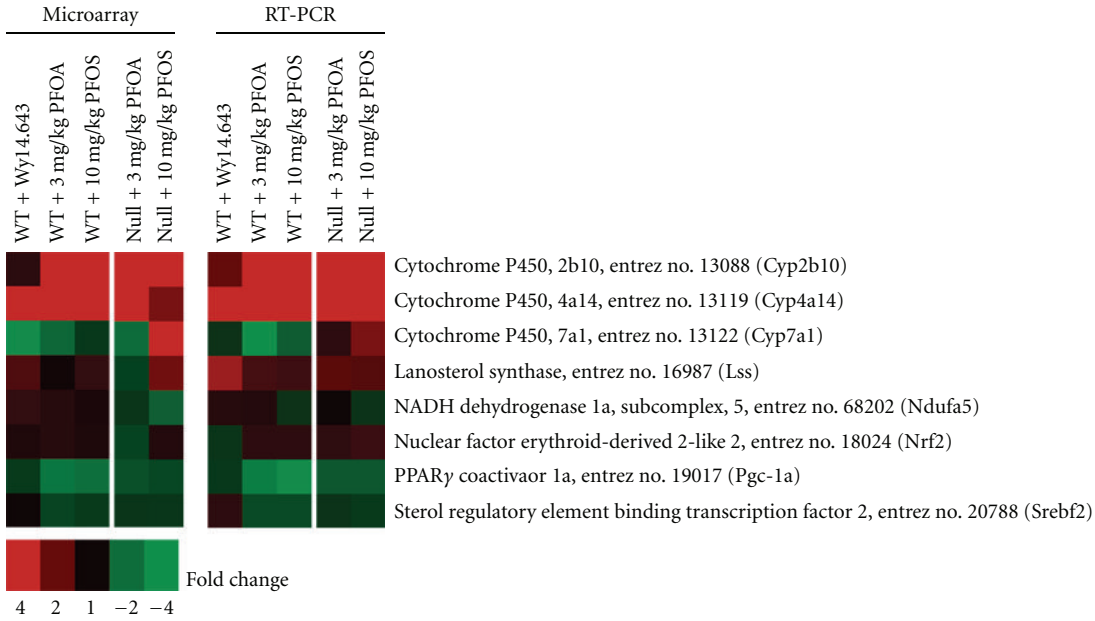

Figure 4: Microarray and Real-time PCR analysis of selected genes. Data from both assays were in close agreement. Small changes in *Ndufa5* expression, a gene which encodes for a subunit of mitochondrial respiratory chain complex I, could not be confirmed by RT-PCR. As predicted based on microarray analysis, PFOS did not appear to up-regulate the expression of *Srebf2*, *Ppargc1a (Pgc-1a)*, or *Nfe2l2* (*Nrf2*) in WT or Null mice. Red or green correspond to average up- or down- regulation, respectively.

stressed, however, that those changes associated with the inflammatory response in Null mice were modest and were only apparent within the context of similar but more robust changes in WT mice. Several categories of genes were also uniquely regulated in Null mice by PFOS including up-regulation of genes in the cholesterol biosynthesis pathway, along with modest down-regulation of genes (<1.5 fold change) associated with oxidative phosphorylation and ribosome biogenesis (Figure 3). Changes related to ribosome biogenesis were particularly subtle and were identified by the computational method provided by GSEA using the complete set of expressed genes without statistical filtering. This approach allowed for an a priori set of genes to be evaluated for significant enrichment without regard for the statistical significance of individual genes. Among the changes uniquely induced by PFOS in Null mice was up-regulation of *Cyp7a1*, an important gene related to bile acid/cholesterol homeostasis. Data for individual genes are provided in Tables 3–10.

3.3. PCR Confirmation. The results from real-time RT-PCR analysis of selected genes are summarized, along with the corresponding results from the microarray analysis, in Figure 4. The data from both assays were in close agreement. It should be pointed out that while up-regulation of *Cyp2b10* was confirmed in treated WT and Null mice, it remained a low copy number transcript in these animals. Down-regulation of *Ndufa5*, a gene which encodes for a subunit of mitochondrial respiratory chain complex I, could not be confirmed in treated Null mice. This result, however, was not surprising because the changes associated with oxidative phosphorylation in the current study were small and, therefore, difficult to detect given the technical variation normally associated with real-time PCR. As predicted based on the microarray results, PFOS did not appear to up-regulate the expression of *Srebf2*, *Ppargc1a*, or *Nfe2l2* (*Nrf2*) in either WT or Null mice.

4. Discussion

In the current study, exposure to PFOS induced both PPARα-dependent and PPARα-independent effects in the murine liver. In WT mice, the observed changes were primarily indicative of a weak PPARα activator. As such, PFOS induced hepatomegaly and altered the expression of genes related to a number of biological functions known to be regulated by PPARα including lipid metabolism, peroxisome biogenesis, proteasome activation, and the inflammatory response [41–45]. These data are also in agreement with previous studies done in either the adult or fetal rodent [46–50]. Among those effects found to be independent of PPARα was altered expression of genes associated with xenobiotic metabolism, including up-regulation of the CAR inducible gene, *Cyp2b10*. Such changes, which were found in both WT and Null mice, were also consistent with results previously reported by our group for PFOA [32, 33]. Although xenobiotic metabolism can be regulated by more than one nuclear receptor [51], the ability of PFOA or perfluorodecanoic acid (PFDA) to activate CAR has been demonstrated in experiments using multiple receptor-null mouse models [31]; therefore, it is likely that PFOS functions as an activator of CAR as well. Additional PPARα-unrelated effects were further indicated

by regulation of a group of genes associated with lipid metabolism and inflammation in both WT and Null mice. As suggested for mice exposed to PFOA [1, 33], such changes could be due to activation of either PPARγ and/or PPARβ/δ. Indeed, studies done using transient transfection reporter cell assays indicate that PFOS and PFOA have the potential to modestly activate other PPAR isotypes. [39, 40]. Furthermore, peroxisome proliferation, a hallmark of PPARα transactivation, can also be induced in the rodent liver by activating PPARγ and/or PPARβ/δ [52]; hence, a degree of functional overlap might be expected among the PPAR isotypes. Particularly noteworthy were PPARα-independent effects that were unique to Null mice since they were not previously observed in mice treated with PFOA [1, 33]. These included modified expression of genes associated with ribosome biogenesis, oxidative phosphorylation, and cholesterol biosynthesis. While activation of PPARα has been linked to changes in cholesterol homeostasis [19] and oxidative phosphorylation [53], it should be stressed that such changes were not simply the result of targeted disruption of PPARα because they were observed in treated animals over and above those effects which occurred in Null controls. Moreover, in the current study, genes linked to cholesterol biosynthesis were found to be up-regulated in Null mice, an effect that mirrored changes previously reported in WT mice treated with the PPARα agonist, Wy 14,643 [1].

Recognition that PPAR ligands can induce "off-target" effects is not new (for review, see [54]). It is not clear, however, whether the effects described for Null mice in the current study were the result of modified activity of transcription regulators, which only became apparent in the absence of PPARα signaling, or whether these changes represent some other aspect of murine metabolism affected by PFOS. Of interest was up-regulation of *Cyp7a1*. This gene encodes for an enzyme responsible for the rate limiting step in the classical pathway of hepatic bile acid biosynthesis and is important for bile acid/cholesterol homeostasis [55]. While targeted disruption of PPARα does not appear to alter basal levels of *Cyp7a1* [56], PPARα agonists such as, fibrates can reduce both *Cyp7a1* gene expression and bile acid biosynthesis in wild-type rodents [57] possibly by interfering with promoter binding of HNF4 [58]. Regulation of *Cyp7a1* is often associated with the liver X receptor (LXR) [59] but it is tightly controlled by multiple pathways and may be positively regulated by the pregnane X receptor (PXR) [60] and the retinoid X receptor (RXR) as well [61]. While the two LXR subtypes, LXRα and LXRβ, are lipogenic and play a key role in regulating cholesterol homeostasis [62, 63], they are not thought to be positive regulators of genes in the cholesterol biosynthesis pathway [64].

Additional signaling pathways that may contribute to the effects observed in Null mice include pathways regulated by Srebf2 (Srebp2) and PPARGC1α (PGC-1α). Srebf2 is one member of a group of membrane-bound transcription factors that play an important role in maintaining lipid homeostasis. SREBF2 is best known for positively regulating cholesterol synthesis in the liver and other tissues (Horton et al., 1998). While decreased nuclear abundance of SREBP2 has been linked to increased hepatic PPARα activity in rats [65], a PPARα-independent mechanism of action has been suggested in mice as well which, in combination with increased expression of CYP7a1, may paradoxically also function via decreased SREBF2 signaling [66]. It should be noted that transcript levels of *Srebf2* were not affected in the current study nor was PFOS found to alter *Srebf2* expression in cultured chicken hepatocytes [67], although such changes are not necessarily required for transcription factor regulation. Rather than functioning as a transcription factor like SREBP2, PPARGC-1α is a transcription coactivator that was first described as a moderator of PPARγ-induced adaptive thermogenesis in brown adipose tissue [68]. PPARGC-1α is now known to regulate various aspects of energy metabolism in different tissues by interacting with a host of transcription factors, including PPARα [69, 70]. Certain PPAR ligands have been shown to inhibit oxidative phosphorylation [71–74] and Walters et al. [75] recently reported that high doses of PFOA could modify mitochondrial function in rats via a pathway involving PPARGC-1α. Unlike their results, however, PFOS did not induce a change in expression of *Ppargc* -1α or its downstream target, *Nrf2*, in the current study. Cellular regulation of metabolism, however, is complex and there are a number of potentially interrelated signaling pathways, including HNF4α [76] and TOR [77], that based on their biological function could theoretically be linked to the effects observed in PFOS-treated Null mice. Given the diversity of effects observed in the current study, it is likely that more than one signaling pathway is responsible for the biological activity reported for PFOS.

Because certain effects were found only in Null mice, their relevance to the toxicity of PFOS is not clear. Although the developmental toxicity of PFOS has been shown to be independent of PPARα in murine neonates [34], it has also been suggested that rather than causing primary alterations to the murine transcriptome, PFOS may alter the physicochemical properties of fetal lung surfactant as the critical event related to toxicity in these animals [78–80]. It should also be stressed that in Null animals the magnitude of change found for certain effects was small, hence, the reported effects in the current study were subtle. On the other hand, these data serve to reinforce two recurring themes regarding the biological activity of PFAAs. First, as a class of compounds, the activity of PFAAs may be quite variable. Differences exist among PFAAs with regard to chain length and functional group which influence, not only the elimination half-life of assorted PFAAs [4, 7] and their ability to activate PPARα [18], but potentially their ability to modify the function of other transcription regulators as well. Second, the biological activity of PFAAs is likely to differ from that observed for fibrate pharmaceuticals, the most commonly studied ligands of PPARα. While much has been learned from studies using fibrate-exposed PPARα-null and PPARα-humanized mice regarding the relevance of chronic PPARα activation to liver tumor formation in humans [22], additional information concerning the biological activity of specific PFAAs remains relevant for risk assessment.

In summary, PFOS is a PPARα agonist that is capable of inducing a variety of PPARα-independent effects in WT

and Null mice, although the toxicological relevance of these changes is uncertain. A number of these effects such as, altered expression of genes involved in lipid metabolism, inflammation, and xenobiotic metabolism were observed in both WT and Null animals, and were consistent with prior studies done with either PFOS or PFOA. Other effects involving genes associated with ribosome biogenesis, oxidative phosphorylation, and cholesterol biosynthesis were unique to Null mice and may represent targeted signaling pathways not yet described for certain PFAAs.

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Research Article

Regulation of Proteome Maintenance Gene Expression by Activators of Peroxisome Proliferator-Activated Receptor α

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The nuclear receptor peroxisome proliferator-activated receptor α (PPARα) is activated by a large number of xenobiotic and hypolipidemic compounds called peroxisome proliferator chemicals (PPCs). One agonist of PPARα (WY-14,643) regulates responses in the mouse liver to chemical stress in part by altering expression of genes involved in proteome maintenance (PM) including protein chaperones in the heat shock protein (*Hsp*) family and proteasomal genes (*Psm*) involved in proteolysis. We hypothesized that other PPARα activators including diverse hypolipidemic and xenobiotic compounds also regulate PM genes in the rat and mouse liver. We examined the expression of PM genes in rat and mouse liver after exposure to 7 different PPCs (WY-14,643, clofibrate, fenofibrate, valproic acid, di-(2-ethylhexyl) phthalate, perfluorooctanoic acid, and perfluorooctane sulfonate) using Affymetrix microarrays. In rats and mice, 174 or 380 PM genes, respectively, were regulated by at least one PPC. The transcriptional changes were, for the most part, dependent on PPARα, as most changes were not observed in similarly treated PPARα-null mice and the changes were not consistently observed in rats treated with activators of the nuclear receptors CAR or PXR. In rats and mice, PM gene expression exhibited differences compared to typical direct targets of PPARα (e.g., *Cyp4a* family members). PM gene expression was usually delayed and in some cases, it was transient. Dose-response characterization of protein expression showed that Hsp86 and Hsp110 proteins were induced only at higher doses. These studies demonstrate that PPARα, activated by diverse PPC, regulates the expression of a large number of genes involved in protein folding and degradation and support an expanded role for PPARα in the regulation of genes that protect the proteome.

1. Introduction

Peroxisome proliferator chemicals (PPCs) are a large class of structurally heterogeneous pharmaceutical and industrial chemicals originally identified as inducers of the size and number of peroxisomes in rodent livers. The peroxisome proliferator-activated receptor family is a subset of the nuclear receptor superfamily and includes three family members (PPARα, β, and γ). The PPARα subtype plays a dominant role in mediating the effects of hypolipidemic and xenobiotic PPC in the liver [1]. Activation of PPARα results in a predictable set of responses in the livers of rats and mice, including hepatocyte peroxisome proliferation, hepatomegaly, hepatocyte hyperplasia, and increased incidence of liver tumors [2]. These responses require a functional PPARα, since PPARα-null mice exposed to the PPARα agonists WY or bezafibrate lack all of these responses [3–5]. PPARα controls these phenotypic responses by regulating a large number of genes in the liver including those involved in lipid homeostasis such as fatty acid oxidation and peroxisome assembly.

Various physical or chemical stressors can produce disease states in which proteins are damaged or misfolded in part through increases in oxidative stress. Many endogenous

pathways are activated to restore cellular homeostasis, including stabilization of unfolded proteins to prevent aggregation as well as removal of damaged or excess proteins through proteolysis. Stabilization of unfolded proteins is performed by molecular chaperones that assist in the folding of nascent polypeptides. Many chaperone genes exhibit increased expression after exposure to a wide variety of stimuli including chemical exposure or increased temperatures and are thus called heat shock (HS) protein (*Hsp*) genes [6–8]. These proteins play key roles in a number of human diseases [9] and are essential for cellular survival under physical or chemical exposure conditions that increase oxidative stress [10, 11]. Additional guardians of the proteome include the genes encoding components of the proteasome. The proteasome carries out ubiquitin-dependent and -independent proteolysis of damaged proteins [12]. The 26S proteasome consists of a 20S core and two 19S regulatory particles containing a total of 28 subunits. Proteasomal (*Psm*) gene expression can be induced by treating cells with proteasomal inhibitors [13].

There is compelling evidence that PPARα protects multiple tissues from oxidative stress induced by chemical insults [14]. The hypolipidemic drug and PPC, clofibrate, protects the liver from damage from the cytotoxicant acetaminophen in wild-type but not PPARα-null mice [15]. Compared to wild-type mice, untreated PPARα-null mice or primary hepatocytes isolated from PPARα-null mice were more sensitive to carbon tetrachloride-, paraquat- or cadmium-induced toxicity [16]. The beneficial effects of caloric restriction in protecting the liver from cytotoxicant-induced liver injury were shown to depend on PPARα [17]. In the kidney, PPARα-null mice were more sensitive to damage after ischemia-reperfusion injury [18], and prior exposure of wild-type mice to PPC reduces the injury [19]. Our previous microarray studies identified PM genes regulated by the PPARα agonist WY-14,643 (WY) including those involved in protein folding (e.g., *Hsp* genes) as well as ubiquitin-dependent and -independent proteolytic processing through the proteasome (e.g., *Psm* genes) [16]. Altered regulation of these genes by PPC could help to explain why PPC exposure through PPARα helps to protect tissues from environmental stressors.

In the present study, we hypothesized that PPARα activators other than WY also regulate PM genes in the rat and mouse liver. We examined the expression of PM genes in rat and mouse liver after exposure to 7 different PPC (WY, clofibrate (CLO), fenofibrate (FENO), valproic acid (VPA), di-(2-ethylhexyl)phthalate (DEHP), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS)) using Affymetrix microarrays from published studies. We show that both therapeutic and environmentally relevant PPC exposure has a dramatic impact on PM gene expression in the rat and mouse liver. Although most of the changes were PPARα-dependent, there were differences in their temporal and dose-dependent regulation compared to typical PPARα target genes involved in fatty acid oxidation. Our findings suggest PPARα is a major regulator of PM genes that have an impact on stress responses in the liver.

2. Materials and Methods

2.1. Animal Studies for Chaperone Protein Expression. The first study was carried out at CIIT, Centers for Health Research, Research Triangle Park, NC utilized wild-type and PPARα-null male mice 9–12 weeks of age on a mixed SV129/C57BL/6J background and have been described previously [20]. Control and treated mice were provided with NIH-07 rodent chow (Zeigler Brothers, Gardeners, PA) and deionized, filtered water *ad libitum*. Lighting was on a 12-hr light/dark cycle. Wild-type and PPARα-null mice were given seven daily gavage doses of 0.1% methyl cellulose control (Sigma Chemical Co., St. Louis, MO), or di-(2-ethylhexyl)phthalate (DEHP) (1000 mg/kg/bw/day) and sacrificed 24 hrs after the last dose.

The second study (NTP study number TOX-60) was carried out at Battelle (Columbus, OH) under a contract from the National Toxicology Program. Male B6C3F1 mice at 4–6 weeks of age were obtained from Taconic Farms, Inc. (Germantown, NY). The feed was NTP-2000 in meal form (Zeigler Brothers, Inc., Gardners, PA) and the drinking water was from the City of Columbus municipal supply. Both feed and water were supplied ad libitum. At 7-8 weeks of age, the mice received in their feed WY at 0, 5, 10, 50, 100, or 500 ppm. The mice were euthanized after 6 days of exposure to WY. Portions of the livers were rapidly snap-frozen in liquid nitrogen and stored at -70°C until analysis. All animal studies were conducted under federal guidelines for the use and care of laboratory animals and were approved by Institutional Animal Care and Use Committees.

2.2. Animal Studies Used for Microarray Analysis. The experiments related to clofibrate- (CLO-) and valproic acid- (VPA-) treated rats were described in Jolly et al. [21]. Briefly, male Sprague-Dawley rats ($n = 5$) were given a single dose of CLO or VPA at the level of 1,000 mg/kg and 2,000 mg/kg, respectively. Animals were killed at 4, 24, and 48 hrs after exposure. Eleven-to-12-week-old male Sprague-Dawley rats were dosed with 20 or 10 mg/kg/day PFOA or PFOS, respectively, in an aqueous solution of 15% Alkamuls EL-620 for 2 days and sacrificed 24 hours later as described in Martin et al. [22]. The animal study of fenofibrate (FENO) was described in Sanderson et al. [23]. Male pure-bred SV129 and PPARα-null mice (2–6 months of age) on a SV129 background were used in the experiments. Fenofibrate was given by gavage (10 mg/ml suspension in 0.5% carboxymethyl cellulose). Animals were sacrificed 6 hours after treatment. Four wild-type and PPARα-null male mice (129S1/SvlmJ wild-type and PPARα-null) per group (6 months of age) were dosed by gavage for 7 consecutive days with PFOA (3 mg/kg/day) in distilled water as described in Rosen et al. [24]. At the end of the dosing period, animals were euthanized by CO_2 asphyxiation and liver tissue was collected for preparation of total RNA.

2.3. Western Blot Analysis. Liver lysates were prepared in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA

with protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin) as previously described [25]. Fifty μg hepatocyte whole cell lysate was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membranes. Immunoblots were developed using primary antibodies against acyl-CoA oxidase (ACO) (a kind gift from Stefan Alexson, Huddinge University Hospital, Huddinge, Sweden), HS proteins (Santa Cruz Biotechnology, Santa Cruz, CA; StressGen, Victoria, B.C., Canada) or CYP4A (GenTest, Waltham, MA) and appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) in the presence of chemiluminescent substrate ECL (Amersham, Piscataway, NJ). Blots were quantitated using Gel-Pro (MediaCybernetics, Silver Spring, MD). Most antibodies recognized only one major band with the expected size. Antibodies to TCP1η routinely gave 2 bands: a ~60 kDa representing the full-length protein and a possible fragment of ~40 kDa. In this study we report the levels of the full-length TCPη and ACO protein (ACO-A). The expression of ACO-B protein, the 52 kDa processed form of ACO-A [26] was also measured. There were 3 animals per treatment group. Variability is expressed as standard error of the mean. Means and S.E. ($n = 3$) for western data were calculated by Student's t-test. The level of significance was set at $P \leq .05$.

2.4. Analysis of Microarray Data. A summary of the microarray studies is shown in Table 1. The doses selected in these studies would be expected to elicit close to a maximal transcriptional response. The raw data files analyzed in this project (.cel files from Affymetrix DNA chips) were either downloaded from Gene Expression Omnibus (GEO) or communicated through the original authors. All of the Affymetrix (Santa Clara, CA) .cel files were first analyzed by Bioconductor SimpleAffy to assess data quality [27]. All .cel files passed this QC step. Data (.cel files) were analyzed and statistically filtered using Rosetta Resolver version 7.1 software (Rosetta Inpharmatics, Kirkland, WA). The background correction was done by Resolver's specific data processing pipeline called Affymetrix Rosetta-Intensity Profile Builder. Statistically significant genes were identified using one-way ANOVA with a false discovery rate (Benjamini-Hochberg test) of ≤0.05 followed by a post hoc test (Scheffe) for significance. Fold-change values <1.5 were removed. As most of the experiments in rats used the RG-U34A array, we compared profiles in the RG-U34A annotation file from Affymetrix (http://www.affymetrix.com/analysis/index.affx). We identified probeset IDs (a total of 8799) on the U34A chip that exhibited sequence similarity with those on the RAE230_2 chip using the "good match" comparison and then built fold-change values for those genes from the RAE230_2 chip which were altered significantly. Heat maps were generated using Eisen Lab Cluster and Treeview software (http://rana.lbl.gov/EisenSoftware.htm). A detailed description of each experiment is available through Gene Expression Omnibus at the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/geo/, as accession numbers indicated in Table 1.

PM genes were identified using the following gene ontology identifiers: 0031072:heat shock protein binding, 0006457:protein folding, 009408:response to heat, 0051085 :chaperone cofactor-dependent protein folding, 0006950:response to stress, 0006983:endoplasmic reticulum overload, 0006512:ubiquitin cycle, and 0006511:ubiquitin-dependent protein catabolic process. A number of proteasome genes (*Psm* family) not linked to GO identifiers were also included.

3. Results

Our previous experiments indicated that a PPARα agonist (WY) alters the expression of PM genes in the mouse liver including those involved in protein folding and protein degradation [16]. To determine if other PPARα agonists have similar activities, we examined transcript profiles in rat and mouse liver after treatment with compounds that possess PPARα agonist-like activities including three hypolipidemic compounds (WY, clofibrate (CLO), fenofibrate (FENO)), an antiepilepsy drug (valproic acid, VPA), and three environmentally relevant chemicals (the plasticizer, di-(2-ethylhexyl)phthalate (DEHP) and the surfactant processing aids, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) (Table 1). PM genes were identified using gene ontology (GO) identifiers (e.g., protein folding; response to stress including endoplasmic reticulum overload; ubiquitin-dependent protein catabolic process).

3.1. Altered Expression of Proteome Maintenance Genes in the Rat and Mouse Liver after Exposure to Diverse PPC. We examined gene expression in the livers of rats and mice treated with PPC. In both species, PPC increased expression of genes known to be regulated by PPARα including those involved in fatty acid oxidation such as *Cyp4a* family members, acyl-CoA oxidase 1 (*Acox1*) and peroxisome assembly genes, for example, *Pex11a* (Figures 1(a) and 2(a)). The global expression of all PM genes in the rat and mouse liver is shown in Figures 1(b) and 2(b), respectively. Out of a total of 288 PM probe sets identified in the rat, 174 were altered by one or more of the 14 treatment conditions (Supplemental File 1). Likewise, out of the total of 1597 PM probe sets examined in the mouse, 382 were altered by one or more of the 12 treatment conditions (see Supplementary Material available online at doi:10.1155/2010/727194).

There were a number of similarities exhibited by the PM genes in both species. First, the altered genes were dominated by those that were upregulated after exposure. The upregulated genes outnumbered the downregulated genes ~2 to 1. Second, the PM genes exhibited a delay in altered expression compared to the known direct targets of PPARα. The direct targets of PPARα were increased as soon as 4 hrs (rat) or 6 hr (mouse) after initial exposure. In contrast, almost none of the PM genes in rats were altered at 4 hrs and some required up to 2 days of exposure before expression changes were observed. Most of the expression changes that occurred after WY exposure in the mouse liver were observed at 5 days but not 6 hrs. Third, a number of PM genes exhibited transient changes compared to

Table 1: Characteristics of the studies used in the rat and mouse liver microarray analysis.

Species	Reference	GEO Accession number	Strain	Dose frequency	Chemical and (Dose)[1]	Time of treatment	Vehicle	Array type	Number of biological replicates	Total number of .cel files
Rat	Martin et al., 2007 [22]	GSE14712	SD (Wistar)	Once daily	PFOA (20) and PFOS (10)	3 days	15% Alkamuls	RAE230 2.0	3	9
Rat	Ellinger-Ziegelbauer et al., 2005 [28]	GSE14712	SD (Wistar)	Once daily	WY (60)	1 day, 3 days, 7 days	Carboxymethylcellulose	RAE230A	3	12
Rat	Jolly et al., 2005 [21]	GSE2303	SD	Once	DEHP (20,000) Valproic acid (2000)	4, 24, 48 hrs	Distilled water	Rat U34A	3–5	16 (DEHP) 15 (VPA)
Rat	Jolly et al., 2005 [21]	GSE2303	SD	Once	Clofibrate (1000)	4, 24, 48 hrs	0.9% saline	Rat U34A	3–5	16
Rat	Nelson et al., 2006 [29]	GSE14712	SD	Once daily	PB (100) and PCN (100)	6 hrs, 1 day, 5 days	1% Tween-80 in 0.5% aqueous methylcellulose	RAE230 2.0	4	36
Mouse	Sanderson et al., 2008 [23]	GSE8292, GSE8295	SV129 and PPARα-null	Once daily	WY[2]	6 hrs, 5 days		Mouse 430_2	3–5 (6 hrs) 4 (5 days)	17 (6 hrs) 16 (5 days)
Mouse	Currie et al., 2005 [30]	Not submitted	C57BL/6J	Once daily	DEHP (1150)	2, 8, 24 hrs, 3 days	Corn oil	Mouse 430_2	3	24
Mouse	Rosen et al., 2008 [24]	GSE9786	SV129 and PPARα-null	Once daily	PFOA (3)	7 days	Distilled water	Mouse 430_2	4	16
Mouse	Sanderson et al., 2008 [23]	GSE8295 GSE8396	SV129 and PPARα-null	Once	Fenofibrate[2]	6 hrs		430_2	4	16

All exposure experiments were conducted on male rats or mice by gavage.

[1]All doses are in mg/kg/day.

Abbreviations: DEHP: di-2-ethylhexyl phthalate; WY: WY-14,643; PFOA: perfluorooctanoic acid; PFOS: perfluorooctane sulfonate; SD: Sprague-Dawley; PB: phenobarbital; PCN: pregnenolone-16alpha-carbonitrile.

[2]400 μL of a 10 mg/mL solution/day.

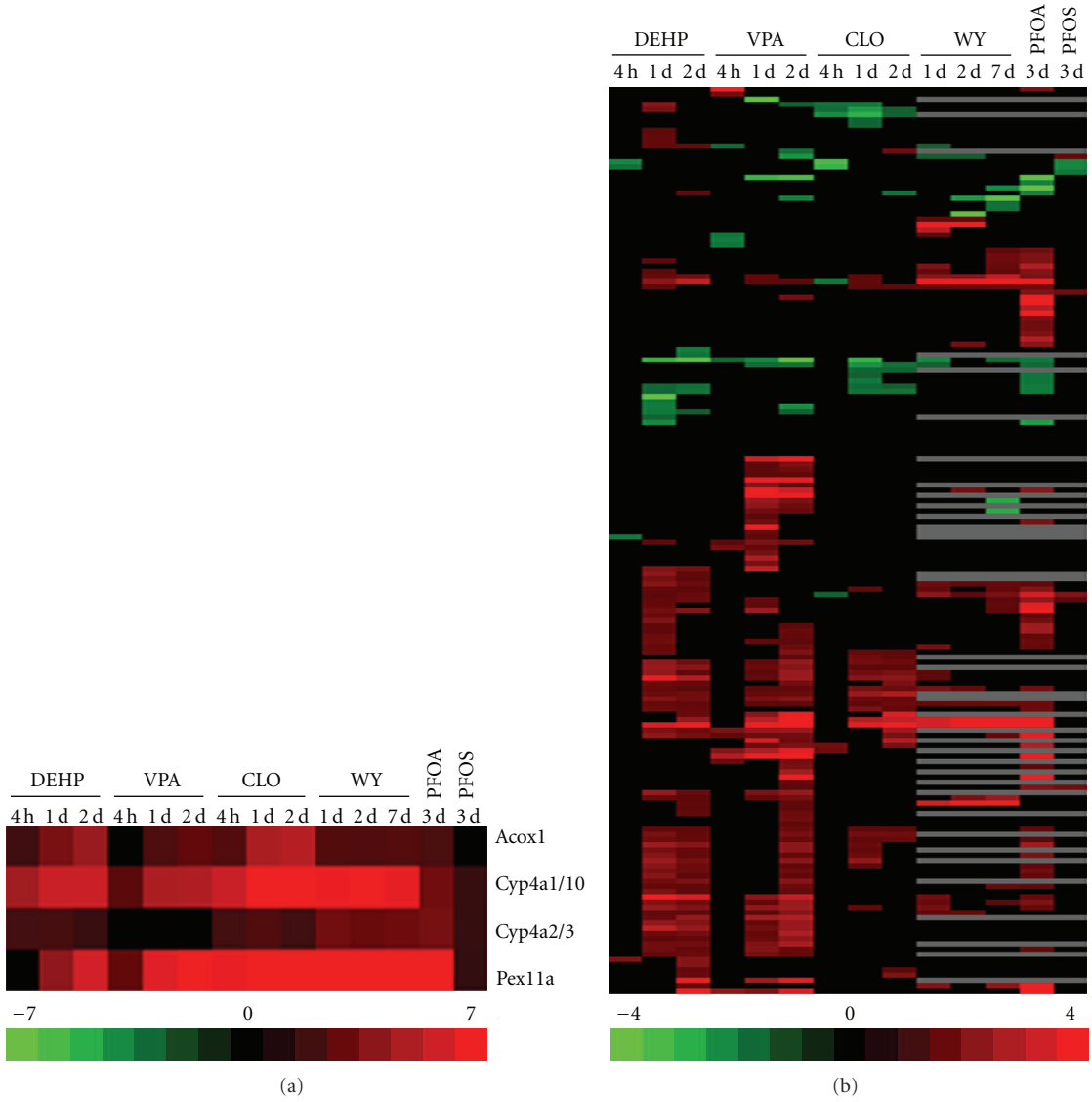

Figure 1: Altered expression of proteome maintenance genes by diverse PPC in rat liver. Male rats were treated with DEHP, VPA, CLO, WY, PFOA, or PFOS for the indicated times. Hepatic mRNA transcripts were assessed using Affymetrix arrays. Genes involved in PM including protein chaperones were identified as described in Section 2. (a) Positive control genes. (b) Expression of all PM genes altered by at least one of the treatments. Genes were clustered using one-dimensional hierarchical clustering. All genes are found in the Supplementary Material. Red: up-regulation; green: down-regulation; black: no change; grey: no data. The intensity scales indicates fold-change due to chemical exposure relative to controls. Abbreviations: WY: WY-14,643; DEHP: di-(2-ethylhexyl)phthalate; PFOA: perfluorooctanoic acid; VPA: valproic acid; CLO: clofibrate; PFOS: perfluorooctane sulfonate.

the constant expression of the direct targets of PPARα. Genes were induced by DEHP, VPA or CLO at 1 but not 2 days; in the mouse liver, a subset of genes were induced by DEHP at 8 hrs but not at any other time. Fourth, there were PPC-specific effects. In the rat VPA, WY and PFOA all increased unique sets of genes. Another set of genes was increased by DEHP at day 1 but decreased by VPA and CLO. In the mouse liver WY, DEHP and FENO altered unique subsets of genes.

The transcriptional changes were, for the most part dependent on PPARα, as most changes were not observed in similarly treated PPARα-null mice. The exceptions included the altered regulation of 6 genes by WY for 5 days, 1 gene by PFOA for 7 days and 41 genes by fenofibrate for 6 hrs. Although fenofibrate is considered a PPARα agonist, there is evidence that other fibrates can activate PPARγ in transactivation assays [1], and pan-PPAR activation may contribute to PPARα-independent regulation of a subset of the PM genes. Overall, these results indicate that both fatty acid metabolizing and PM genes were dependent on PPARα for altered regulation by PPC. The PM genes exhibited unique characteristics in their pattern of expression.

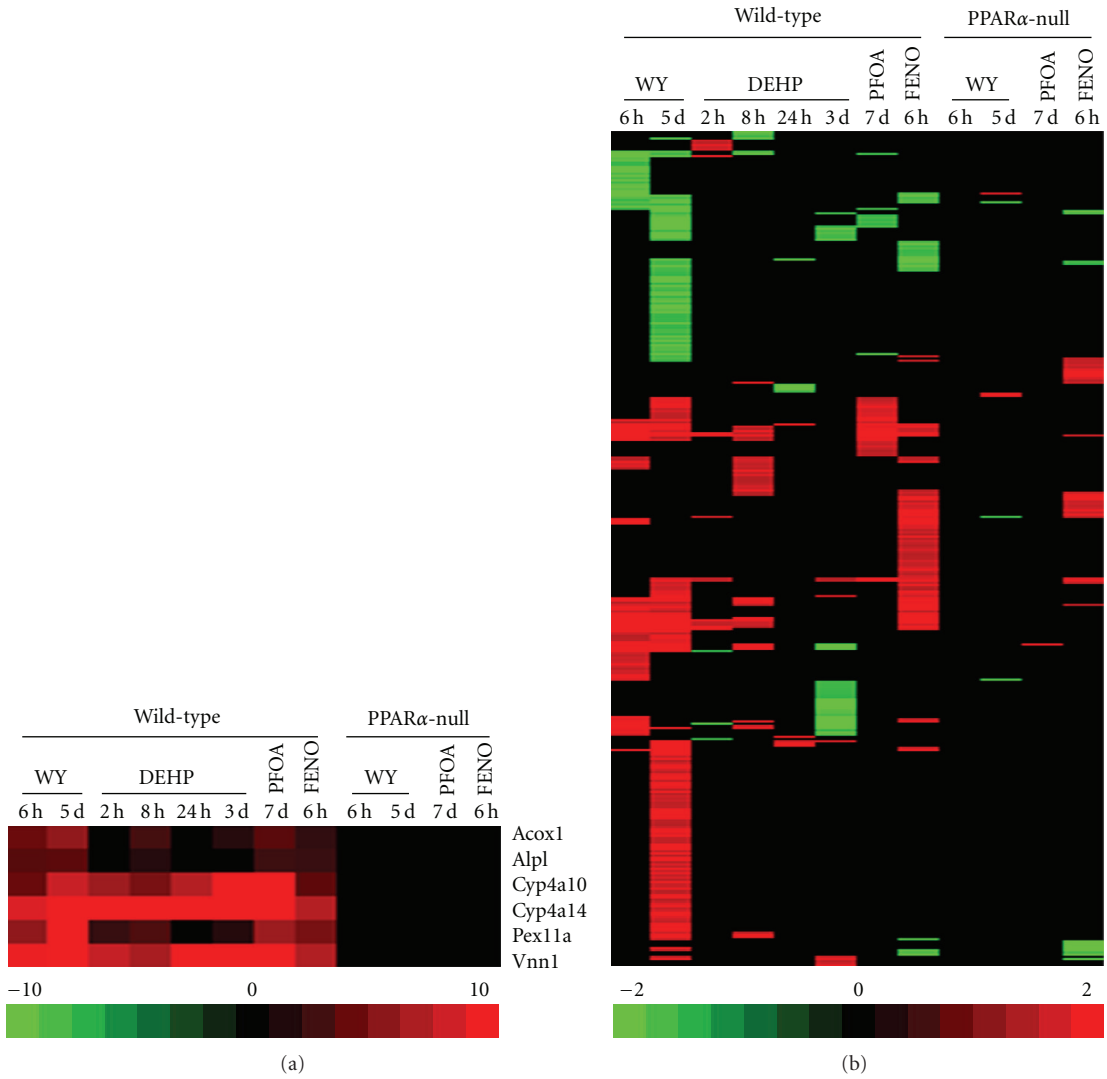

Figure 2: Altered expression of proteome maintenance genes by diverse PPC is predominantly PPARα dependent. Wild-type or PPARα-null mice were treated with WY, DEHP, PFOA, or FENO for the indicated times. Hepatic mRNA transcripts were assessed using full-genome Affymetrix arrays. Genes involved in PM including protein chaperones were identified as described in Section 2. (a) Positive control genes. (b) Expression of all PM genes altered by at least one of the treatments. Genes were clustered using one-dimensional hierarchical clustering. All genes are found in Supplemental Material. Red: up-regulation; green: down-regulation; black: no change. The intensity scales indicate fold-change due to chemical exposure relative to controls. Abbreviations: WY: WY-14,643; DEHP: di-(2-ethylhexyl)phthalate; PFOA: perfluorooctanoic acid; FENO: fenofibrate.

3.2. Regulation of Proteasomal Genes by PPC. A large number of genes encoding components of the proteasome were altered by WY [16]. We examined the expression of the proteasomal genes (*Psm*) as well as those known to be involved in ubiquitin-dependent proteolysis after PPC exposure (Figures 3(a) and 3(b)). In both species all of the *Psm* genes which exhibited altered regulation were upregulated except those that are components of the immunoproteasome (i.e., *Psmb8* in rats and *Psmb8, Psmb9* and *Psmb10* in mice). DEHP and VPA in rats and WY and PFOA in mice altered the largest number of *Psm* genes. In contrast, DEHP in mice transiently altered only a small set of *Psm* genes. The changes were never observed earlier than 1 day in rats. DEHP, VPA and PFOA in rats and WY and PFOA in mice altered subunits of both the catalytic core (20S proteasome) and the ATP-dependent regulatory core (19S proteasome), whereas CLO and WY in rats preferentially altered 20S components. There were a number of genes that were altered in both species including *Psma1, Psma5, Psma7, Psmb2, Psmb3, Psmb4, Psmb8, Psmc4, Psmd1, Psmd4,* and *Psmd13*. All of the *Psm* genes altered by PPC in mice were PPARα dependent.

In an examination of the ubiquitination machinery, PPC altered 8 probesets (6 genes) in rat liver and 48 probesets (35 genes) in mouse liver (data not shown; see Supplemenary Material). None of the rat genes were altered by more than two PPC and in the mouse liver, only one gene (*Usp38*) was altered by three out of the four PPCs. All but six of the ubiquitination genes were PPARα dependent.

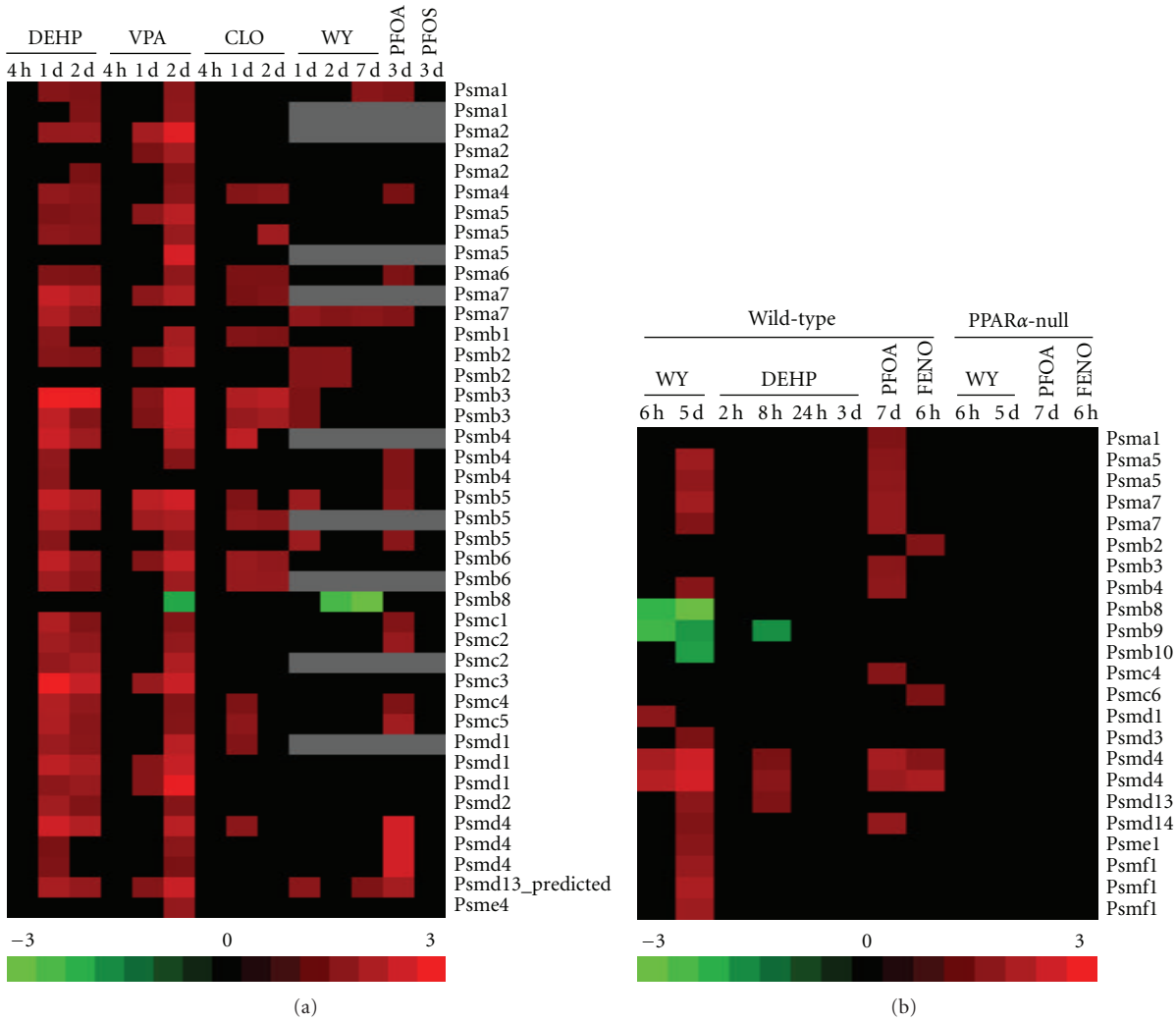

Figure 3: Expression of the proteasomal genes in the rat and mouse liver after PPC treatment. Proteasomal gene expression was examined in the (a) rat liver or (b) mouse liver after PPC exposure using the studies described in Figures 1 and 2. Genes which exhibited altered expression in at least one of the treatments are shown. Genes are presented in alphabetical order. Many genes were represented by more than one probe set.

These findings extend the results from our earlier study and show that diverse PPCs coordinately alter the expression of the *Psm* genes in a PPARα-dependent manner.

3.3. Regulation of Protein Chaperone Genes by PPC. We examined the expression of protein chaperone genes after PPC exposure (Figures 4(a) and 4(b)). Almost all of the chaperone genes were upregulated by PPC in rat liver and by WY in the mouse liver. There were many genes that were regulated by more than half of the PPC in rat liver including *Hsp90aa1, Hsp90ab1, Hspa8, Hspb1, Hspd1, Hspe1, Hspa9,* and *Hsph1*. In the mouse liver most of the PPC regulated a smaller set of genes including *Dnaja2, Grpel2, Hsp90aa1, Hspa4l, Hspa8, Hspb1,* and *Hsph1*. Many genes were commonly regulated in both species by at least half of the PPC (*Dnaja1, Dnaja2, Hsp90aa1, Hspa1a/b, Hspa8, Hspb1, Hspd1, Hspe1, Hspa9,* and *Hsph1*). Some of the changes were transient, exhibiting attenuated or no regulation after long-term exposure. For WY (Figure 4(b)), these included *Dnaja1, Dnajb1, Dnajb4, Hspa1a, Hspa1b, Hspa8, Hsph1*, and *Hsp90aa1* and for DEHP, these included *Dnaja1, Dnaja2, Dnajc12, Grpel2, Hsp90aa1, Hspb1,* and *Hspb8*. There were a number of chaperone genes that were uniquely regulated by VPA (*Dnaja4, Hspa4*), PFOA (*Hspa9a-predicted*) or both (*Hspa1b*) in rat liver. Exposure to DEHP in the mouse for 3 days gave a unique pattern of changes in which *Dnaja1, Dnajb1, Dnajb4, Hspa1a, Hspa1b,* and *Hsph1* were downregulated at 3 days compared to the consistent up-regulation by the other PPC. Furthermore, three of these genes (*Hspa1a, Hspa1b, Hsph1*) were upregulated after shorter-term exposure. These results indicate that multiple and possibly competing mechanisms may be regulating these genes after DEHP exposure, different from that of other PPC. In the mouse liver, most of the genes required PPARα for

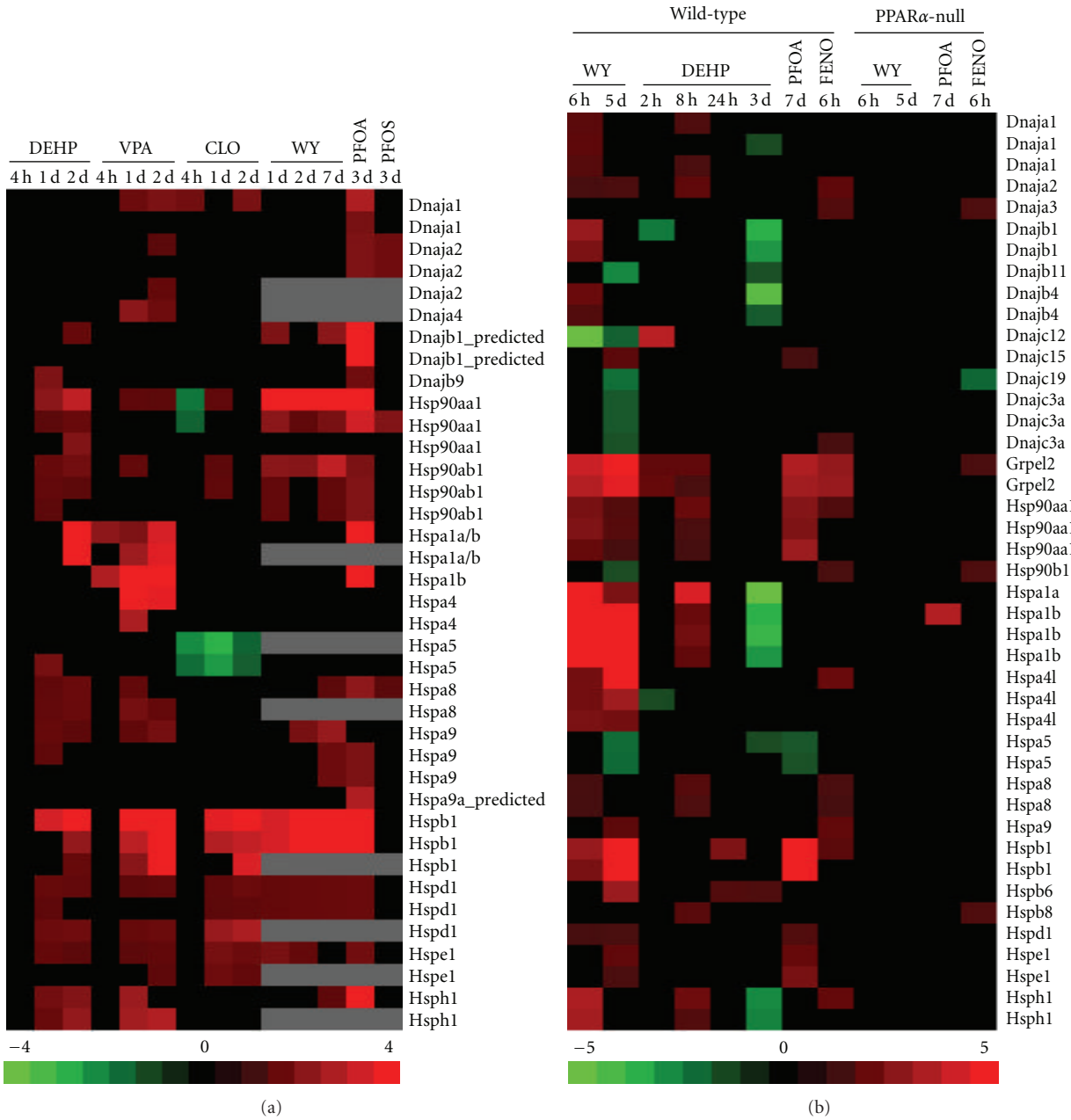

Figure 4: Expression of the protein chaperone genes in the rat and mouse liver after PPC treatment. Protein chaperone gene expression was examined in the (a) rat liver or (b) mouse liver after PPC exposure using the studies described in Figures 1 and 2. Genes which exhibited altered expression in at least one of the treatments are shown. Genes are presented in alphabetical order. Many genes were represented by more than one probe set.

altered expression except for *Dnaja3, Grpel2,* and *Hsp90b1* that appeared to be regulated similarly by FENO in wild-type and PPARα-null mice.

3.4. Expression of Chaperone Proteins in Mouse Liver after PPC Exposure. We examined expression of protein chaperones in protein extracts from livers of mice given five different doses of WY for 6 days or DEHP at one dose level for 7 days. Given the transcriptional increases in chaperonin-containing T-complex 1 (Tcp-1) family members *Cct3, Cct4, Cct7,* and *Cct8* after exposure to PPC in wild-type mice (Supplemental Material), we also examined the expression of Tcp1η protein. Dose-dependent increases in Tcp1η, Hsp86, Cyp4A, ACO-A and Hsp110 were observed in WY-treated wild-type B6C3F1 mice (Figure 5(a)). The chaperones exhibited dissimilar dose-dependent inductions as TCP1η was induced at lower doses similar to the direct PPARα targets ACO-A and Cyp4a, whereas Hsp86 and Hsp110 were induced only at the higher doses. Induction of Hsp25 and Hsp70 was strain specific; induction by WY was observed in SV129 mice [31]

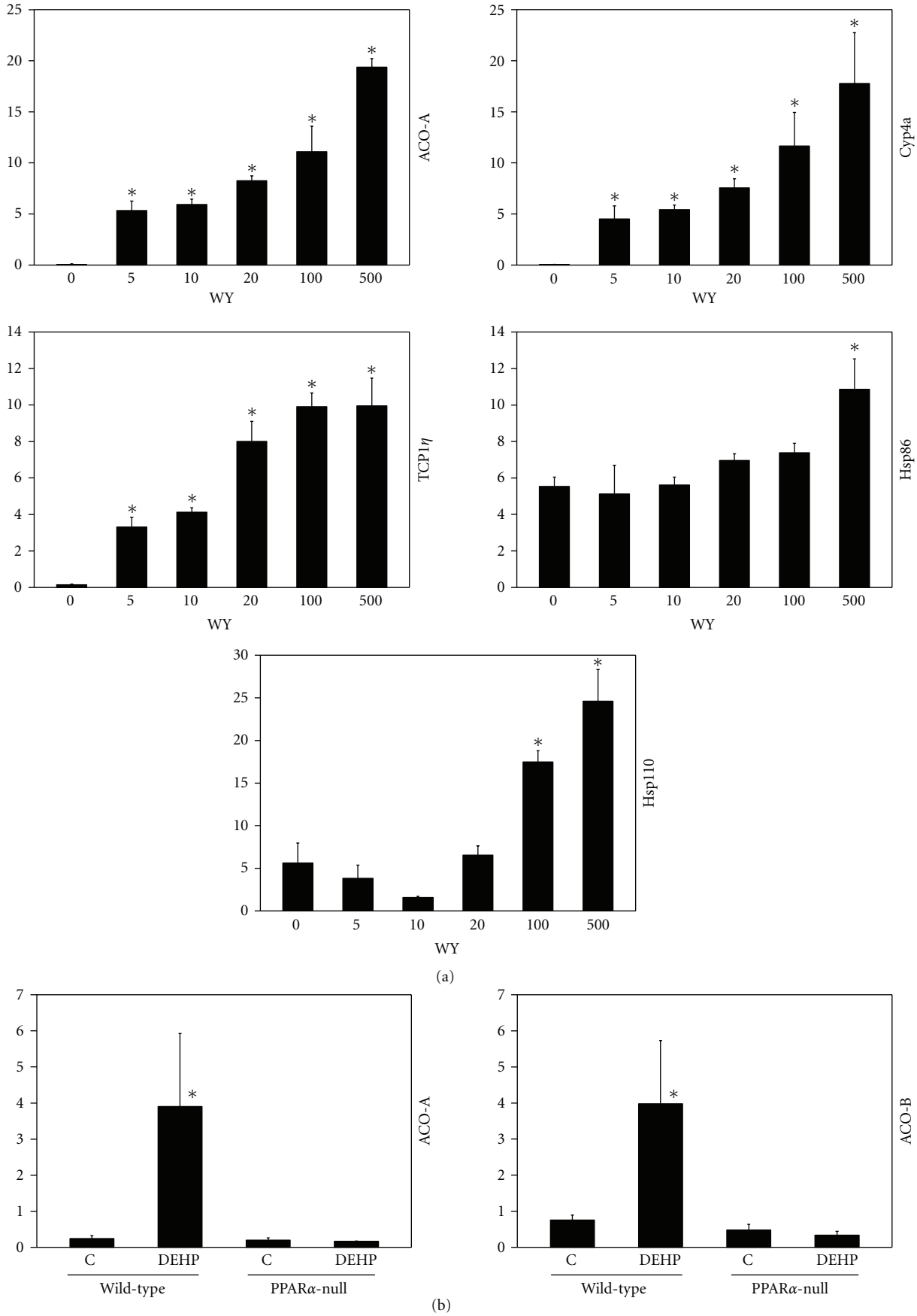

Figure 5: Continued.

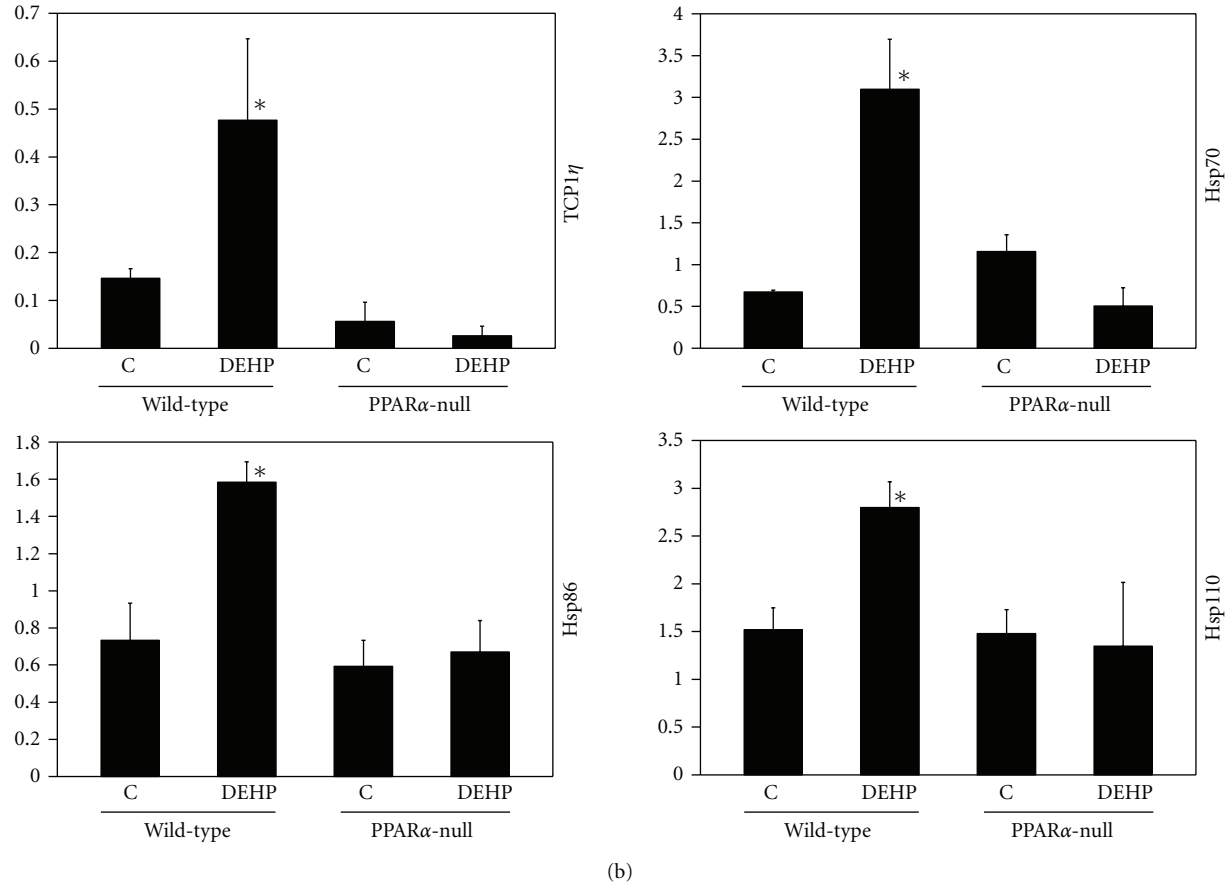

Figure 5: Expression of chaperone proteins after PPC treatment. (a) Protein expression in the livers of mice treated with different doses of WY. B6C3F1 mice were treated with the indicated concentrations of WY in the diet for 6 days. Protein expression was assessed by Westerns using primary antibodies against the indicated proteins. Blots were quantitated as described in Section 2. (b) Protein expression in livers of wild-type and PPARα-null mice after exposure to DEHP. Proteins were extracted from the livers of wild-type or PPARα-null mice given 7 consecutive doses of DEHP (1000 mg/kg day). There were 3 animals per treatment group. Variability is expressed as standard error of the mean. Means and S.E. ($n = 3$) for western data were calculated by Student's t-test. The level of significance was set at $P \leq .05$ and significance is indicated with $*$.

but not in B6C3F1 mice (data not shown). Like ACO-A and ACO-B proteins, induction of Tcp1η, Hsp70, Hsp86, and Hsp110 protein expression was observed after exposure to DEHP in wild-type but not PPARα-null mice (Figure 5(b)). These results demonstrate that diverse PPC induce protein chaperone expression dependent on PPARα.

3.5. Comparison of the Proteome Maintenance Genes Altered by Chemicals That Activate Other Nuclear Receptors. Many of the PM genes have been shown to be induced under conditions of stress leading to the hypothesis that the response to PPC may be due to activation of a generalized stress response. If that was the case, we would predict that the genes would be altered by other chemicals given at relatively high doses. To determine the specificity of the PPC response, the expression changes of the PM genes were compared between PPC and chemicals which activate other nuclear receptors: phenobarbital (PB) which activates CAR and pregnenolone-16alpha-carbonitrile (PCN) which activates the pregnane X receptor (PXR). In a published study [29], PB, or PCN were given to rats at 100 mg/kg/day for 6 hrs, 1 day or 5 days. Microarray analysis was performed using the same microarray platform and analysis procedures as described above. We compared the PM genes and found that out of the genes that were altered by PPC, PB or PCN, most of the genes were uniquely altered by the PPC (Figure 6(a)). In contrast, only 3 genes were altered by PB and/or PCN but not any of the PPC. There were 47 overlapping genes which exhibited for the most part similar expression by the PPC and PB or PCN (Figure 6(b)). In particular, there was a group of genes that were consistently upregulated by PB, PCN and two or more PPC including *Ppil3, Psma2, Psma7, Psmb2, Psmb3, Psmb4, Psmb5, Psmc4,* and *Psmc5*. Due to their promiscuous induction by most of the chemicals, we hypothesize that these genes were altered due to a generalized stress response and not due to activation of a particular nuclear receptor. However, most of the PM genes that were altered by PPC formed a unique group that was only altered by one or more PPC but not by activators of other nuclear receptors.

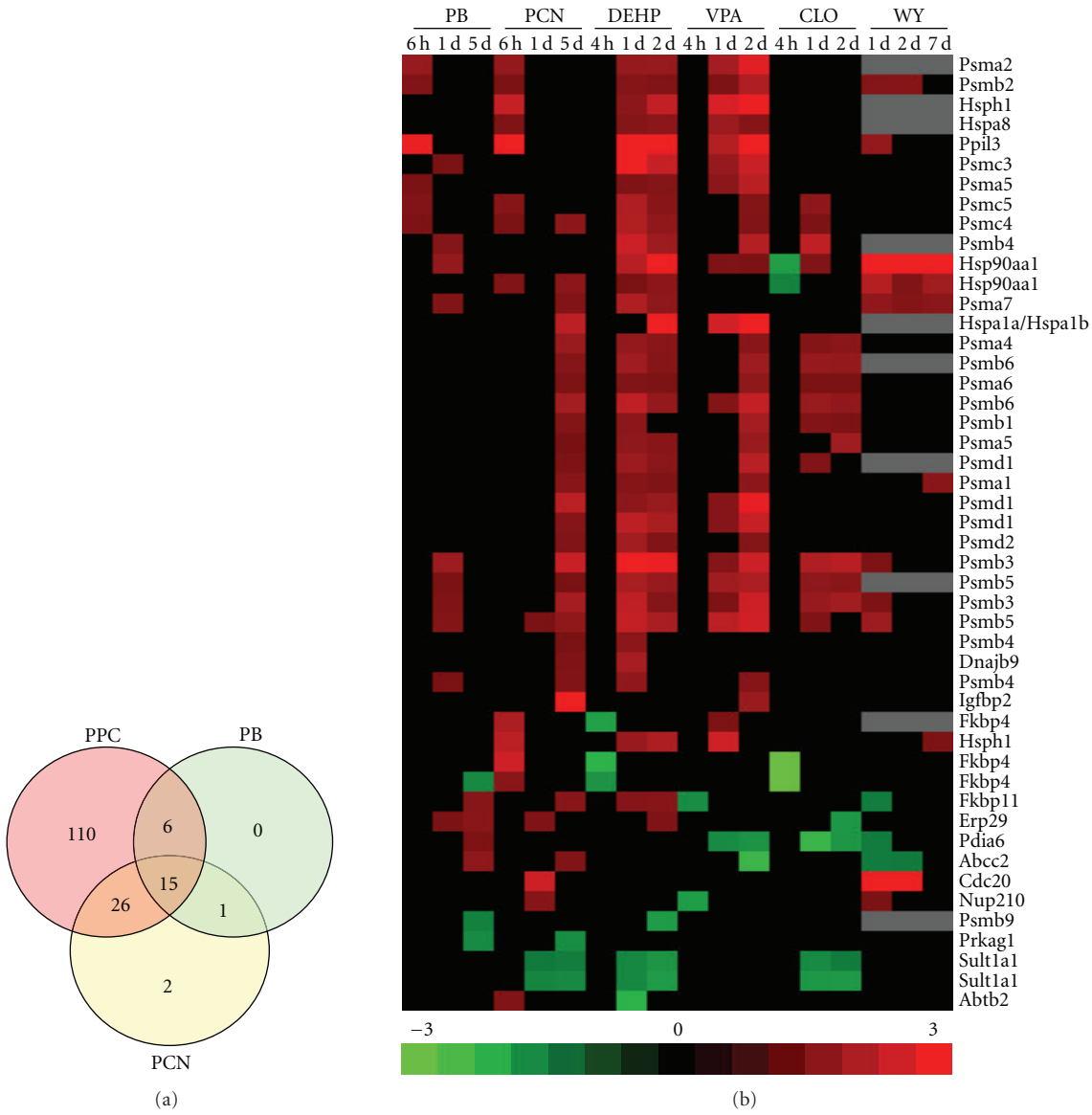

Figure 6: Comparison of the expression of the PM genes after exposure to PPARα, CAR and PXR activators. Lists of differentially regulated genes were generated after exposure to phenobarbital (PB), pregnenolone-16alpha-carbonitrile (PCN) or the PPC. The expression of the PM genes (probe sets) were compared between the treatments. (a) Overlap in the PM gene probesets that were altered in one or more of the three time points after PB or PCN exposure or after 3 or 4 of the 4 PPC treatment groups are shown. (b) The 47 overlapping gene probe sets were clustered as described above.

4. Discussion

The nuclear receptor PPARα is considered a key factor in lipid homeostasis. There is increasing evidence that PPARα plays additional functional roles in the liver by regulating responses to various chemical and physical stressors [14]. An agonist of PPARα (WY) regulates responses in the mouse liver to chemical stress in part by altering expression of genes involved in proteome maintenance (PM) such as the *Hsp* genes involved in protein folding and *Psm* genes involved in proteolysis. In this study, we show that other PPARα activators including diverse hypolipidemic and xenobiotic compounds also regulate a common set of PM genes in the rat and mouse liver. These transcriptional changes were, for the most part dependent on PPARα because most of the altered genes were altered by PPC but not by chemicals that activate other nuclear receptors, CAR and PXR. In mice the changes in the PM genes were observed in wild-type but not PPARα-null mice. The responsive PM genes did not exhibit the same transcriptional behavior as genes known to be direct targets of PPARα (e.g., *Acox1* or *Cyp4a* family members) in which PPARα binds directly to peroxisome proliferator response elements (PPRE) in their promoters. While the direct targets were upregulated early after exposure and remained elevated throughout the duration of the experiment, the PM genes exhibited a lag before expression

changed and in many cases, the changes were transient. A number of PM genes were identified that were not universally regulated by all of the PPC. Discordance in the expression pattern between the PPC could be explained in part due to the selection of dose and time which can both influence the gene expression results of these studies. However, our studies indicate that the PM genes are a unique subset of PPARα target genes that appear to be regulated by a mechanism different than fatty acid oxidation genes.

How are the PM genes regulated by PPC through PPARα? Given that *Hsp* gene expression is controlled in part by heat shock factor 1 (HSF1), one possibility is that the increases in *Hsp* gene expression are secondary to increases in the expression and activity of HSF1. However, we did not observe changes in HSF1 expression in our transcript profiling studies and earlier studies showed that HSF1 and HSF2 expression and binding to HSE were not altered by WY exposure in the rat liver [32]. To help determine whether regulation of *Hsp* gene expression is direct or indirect, we examined their promoters and found that only a few genes possess a putative PPRE(s) (data not shown). The fact that most of the *Hsp* and *Psm* genes are not immediately increased by PPC exposure indicates that additional molecular events are required before induction can occur.

Many *Hsp* genes may be regulated indirectly through generalized stress responses especially those that are induced after exposure to the relatively high doses of the chemicals used in the animal studies. One of the stress responses that may be driving the expression of the genes is increases in oxidative stress. There is abundant evidence for the increased expression of chaperone gene expression by oxidative stress [10, 33]. PPC exposure leads to increases in oxidative stress and lipid peroxidation mediated through increased activities of enzymes that generate reactive oxygen species (reviewed in [2]). Furthermore, as oxidative stress after PPC exposure is a relatively high dose phenomenon [2], the induction of Hsp86 and Hsp110 only at high doses is consistent with an indirect mechanism of induction, possibly through increases in oxidative stress. Likewise, induction of *Psm* gene expression may be an adaptation to decreases in the levels of functional proteasomes through damage by oxidative stress. Treatment of cells *in vitro* with proteasome inhibitors increased the expression of a broad range of subunits of the proteasome in diverse species [13, 34] even when less than 50% of the total proteasomal activity was inhibited [13]. Proteasome inhibition resulted in increased expression of 19S and 20S components but decreased expression of *Psmb8* [13], a pattern similar to that observed with PPC. Since direct oxidative modification of the catalytic core subunits of the proteasome inhibits their activities [35], PPC may be increasing the level of oxidized proteins that inhibit the proteasome, triggering compensatory increases in *Psm* genes. Lastly, the absolute increases in expression of some *Hsp* and *Psm* genes was higher in WY-treated mice nullizygous for Nrf2, a transcription factor activated by oxidative stress that regulates genes that dampen oxidative stress. Thus, in the absence of Nrf2, increased levels of oxidative stress may have contributed to the greater increases in the PM genes by WY [16]. Taken together, these results indicate that PPARα may regulate the PM genes secondary to increases in oxidative stress. An alternative hypothesis is that PM genes are induced in response to the demand for folded proteins under conditions of increased protein synthesis during active reprogramming of gene expression coincident with increases in peroxisomes and smooth endoplasmic reticulum (SER) and increases in cell number. This hypothesis is consistent with the fact that a subset of the PM genes were induced by CAR, PXR as well as PPARα activators, all of which induce SER proliferation and hepatocyte proliferation. This would also help to explain the somewhat transient nature of the gene expression changes as after acute exposure the liver reaches a new equilibrium in which hepatocyte proliferation returns to normal levels. Further experiments are needed to determine the molecular basis for the induction of the PM genes.

What might the increased levels of PM proteins be doing in the liver after WY exposure? Increased levels of PM gene products might allow tight control of the inducibility of PPARα. Many nuclear receptors interact with chaperone proteins including the ones induced by PPC in our studies [36]. PPARα interacts with Hsp72 [37] and is inhibited by Hsp90 [38]. PPARα activation is also downregulated by proteasomal proteolysis [39]. Thus, induction of some PM genes may dampen the PPARα transcriptional response. *Hsp* induction may also help support the increases in protein synthesis required for liver enlargement including peroxisome proliferation after PPC exposure. Increased expression of TCP1 subunits may be important for proper protein insertion into the peroxisomal membrane [40]. Increased expression of *Hsp* family members has been mechanistically linked to protection from apoptosis [41, 42] and PPC, at least under acute exposure conditions, decrease basal levels of apoptosis [2].

A fundamental question arises as to why PPARα would have a dual role of regulating both PM genes involved in stress responses and lipid metabolism genes. The ability of PPARα to act as a regulator of responses to different types of stressors may have coevolved and become inexorably linked with the most important stressor mammals face in the wild, that is, an inadequate or inconsistent food supply. Periods of starvation or caloric restriction requires a reprogramming of gene expression to utilize stored fat and to allow adaptation to new, potentially toxic food sources. PPARα is a master regulator of the starvation response. Gene expression changes by fasting [43, 44] or caloric restriction [14, 17] partly depend on PPARα including genes that mobilize, transport and catabolize fats. The ability of PPARα to also regulate genes (e.g., *Hsp* family members) involved in suppression of cytotoxicity induced by unfolded proteins would make teleological sense and may allow increased resistance to potentially toxic foods animals are forced to eat when the customary foods are no longer available.

In summary, we used transcript profiling to show that PPARα activated by diverse PPC regulates the expression of two classes of genes that may be responsible for protection from chemical-induced oxidative stress: the chaperone genes involved in protein folding and genes involved in proteasomal degradation of damaged proteins. Induction of

these potentially protective pathways may provide efficient means for cells to survive conditions of oxidative stress that contribute to chronic diseases. Induction of these pathways through pharmacological means provides opportunities for protection in a number of settings in which there is induction of oxidative stress, oxidative damage to proteins, and increased occurrence of disease.

Author Contributions

H. Ren analyzed the microarray data and helped draft the paper. B. Vallanat performed the microarray data analysis. H. M. B. -Borg analyzed the western data. R. Currie generated microarray data. J. C. Corton conceived of the study, participated in study design and animal studies, generated western data, analyzed microarray data and helped to draft the paper. All authors read and approved the final paper.

Abbreviations

CLO: clofibrate;
DEHP: di-(2-ethylhexyl)phthalate;
FENO: fenofibrate;
HSF: heat shock factor;
HSP: heat shock proteins;
PCR: polymerase chain reaction;
PFOA: perfluorooctanoic acid;
PFOS: perfluorooctane sulfonate;
PM: protein maintenance;
PPAR: peroxisome proliferator-activated receptor;
PPC: peroxisome proliferator chemical;
PPRE: peroxisome proliferator response element;
Psm: proteasome;
VPA: valproic acid;
WY: WY-14,643.

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Review Article

Gene Expression Changes Induced by PPAR Gamma Agonists in Animal and Human Liver

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Thiazolidinediones are a class of Peroxisome Proliferator Activated Receptor γ (PPARγ) agonists that reduce insulin resistance in type 2 diabetic patients. Although no detectable hepatic toxicity has been evidenced in animal studies during preclinical trials, these molecules have nevertheless induced hepatic adverse effects in some treated patients. The mechanism(s) of hepatotoxicity remains equivocal. Several studies have been conducted using PCR analysis and microarray technology to identify possible target genes and here we review the data obtained from various *in vivo* and *in vitro* experimental models. Although PPARγ is expressed at a much lower level in liver than in adipose tissue, PPARγ agonists exert various PPARγ-dependent effects in liver in addition to PPARγ-independent effects. Differences in effects are dependent on the choice of agonist and experimental conditions in rodent animal studies and in rodent and human liver cell cultures. These effects are much more pronounced in obese and diabetic liver. Moreover, our own recent studies have shown major interindividual variability in the response of primary human hepatocyte populations to troglitazone treatment, supporting the occurrence of hepatotoxicity in only some individuals.

1. Introduction

Obesity has emerged as a major health problem with 1.6 billion adults classified as overweight and obese. The condition is associated with type 2 diabetes, cardiovascular diseases, and several cancers [1] and is characterized by an increase in the size and number of adipocytes. Peroxisome proliferator-activated receptors (PPARs) act as lipid sensors and therefore represent critical molecular targets for the treatment of obesity. Thus, agonists of peroxisome proliferator-activated receptor γ (PPARγ, also known as NR1C3) are used to treat non-insulin-dependent diabetes type 2. PPARγ belongs to the superfamily of nuclear receptors; it acts as a critical transcription factor in the regulation of adipose differentiation, lipid storage, and of genes involved in energy storage and utilisation. One putative mechanism through which PPARγ enhances insulin sensitivity is its ability to channel fatty acids into adipose tissue, thus decreasing plasma fatty acid concentration. PPARγ can also affect insulin sensitivity by regulating hormones, cytokines, and proteins that are involved in insulin resistance [2]. It exists as two forms encoded by multiple transcript variants. PPARγ1 is the predominant isoform in humans; it is highly expressed in adipose tissue but is also expressed in many other cell types in which it plays important functions, particularly intestine and immune cells. PPARγ1 is the main isoform found in liver. PPARγ2 is found at high levels in different adipose tissues [3]. Hepatic PPARγ represents only 10–30%

of the level in adipose tissue [4]. The PPAR superfamily contains two other subtypes, PPARα (NR1C1) and PPARβ/δ (NR1C2). PPARα is highly expressed in liver, kidney, small intestine, heart, and muscle, and it involved in fatty acid catabolism. PPARβ/δ is ubiquitous; although less studied, it is also implicated in fatty acid oxidation [5].

The mechanisms of action of PPARs have been well studied. Following activation by their ligands and heterodimerisation with retinoid X receptor (RXR), PPARs undergo specific conformational changes that release corepressors (as NcoR2/SMRT) and allow for the recruitment of coactivators (as SRC1/NCoA1, TIF2/SRC2, CBP/P300, steroid receptor coactivator 1, RIP140 (receptor interacting protein 140), PPARγ co-activator-1) [6–8]. PPARs then interact with the peroxisome proliferator element (PPRE) in the promoter region of their target genes involved in lipid catabolism, fatty acid transport, and glucose homeostasis [9]. Their differential effects could be explained by the cell and promoter context as well as the availability of cofactors but also by the specific conformation changes of the receptor induced by each PPARγ ligand that leads to differential promoter activation and chromatin remodelling of target genes [10].

A wide variety of natural and synthetic PPARγ ligands have been identified. Besides natural ligands such as 15-deoxy-prostaglandin J2, a metabolite of prostaglandin D2 and vitamin E, PPARγ agonists include several synthetic drug classes such as glitazones and tyrosine analogs. Thiazolidinediones (TZDs) are a class of PPARγ agonists used in clinical practice to reduce plasma glucose level in type 2 diabetic patients. The adipose tissue is required for these agonists to exert their antidiabetic but not their lipidomic effects [11]. TZDs of the first generation were found to be highly hepatotoxic; the first one, ciglitazone (CIG), was abandoned after clinical trials and the second, troglitazone (TRO), was rapidly withdrawn from the market after reports of severe liver failure and death [12]. A second generation of PPARγ agonists, rosiglitazone (ROSI) and pioglitazone (PIO), has been approved by the Food and Drug Administration (FDA) in 1999. Hepatic failures have also been observed after administration of these two TZDs but they were less frequent and severe [12]. The antidiabetic activities of another class of PPARγ agonists, referred as tyrosine analogs, such as GW1929 and GW7845, looked promising but none of these compounds has been released on the market as yet [13].

Since dual PPARα and PPARγ agonists might provide broader beneficial metabolic effects through a simultaneous treatment of hyperglycemia and dyslipidemia, compounds targeting both PPARα and γ have been developed by the pharmaceutical industry. However, the first dual agonists, muraglitazar and tesaglitazar, have been stopped during clinical trials due to cardiac and renal side-effects, respectively [14]. Other molecules are still under development, for example, drugs belonging to a new class called selective PPAR modulators (SPPARM) for the reduction of the side-effects found with glitazones, such as oedema and weight gain [15].

A major concern in the development of novel PPARγ agonists that differ from the current therapeutics is their implication in tumor development in different tissues. Although, whether their activation promotes or limits this process remains unclear and may depend on specific conditions [16], the FDA requires 2-year carcinogenesis studies in rodents of new agonists prior to the commencement of clinical trials exceeding 6 months.

Major species differences exist in the sensitivity to TRO. During preclinical trials, TRO did not induce detectable hepatic toxicity in animals, including monkeys, which show similar metabolic profiles to humans [17], supporting the view that glitazone toxicity is restricted to human individuals having a particular phenotype. Consequently, it could be postulated that the use of human liver cell models represents a more suitable approach than the use of their animal counterparts for investigations of hepatotoxic effects of PPARγ agonists.

Microarray technology represents a powerful tool to better understand the mechanisms of drug toxicity since it permits the identification of gene sets that are preferentially modulated after treatment. Several *in vivo* and *in vitro* studies have already been published on the effects of PPAR agonists on gene expression using different experimental conditions. However, they mainly concern PPARα agonists [18–22]. Studies on PPARγ agonists are limited and are usually focused on nonhepatic tissues, especially adipose tissue. We review here the effects of PPARγ agonists on hepatic gene expression described in the literature using either *in vivo* animal models or *in vitro* animal and human liver cell models and make comparison with our own recent data obtained with human hepatocyte cultures.

2. *In Vivo* Animal Studies

2.1. Effects of PPARγ Agonists in Normal Liver. Little information exists on gene profiling changes induced by PPARγ agonists in the liver of normal animals (Table 1); this might be explained by the low expression of this receptor in this organ. Most studies relate to PPARα agonist effects. However, a comparison of transcriptomic profiles of ROSI with six PPARα agonists has clearly shown that this glitazone does not significantly regulate any of the PPARα target genes in Sprague-Dawley rat liver [23]. In this study, Cyp4a10, a cytochrome P450 involved in lipid metabolism, was induced 14-fold by Wy-14643 (one of the most potent PPARα agonists) and only 1.5-fold by ROSI. According to Memon et al. [24], the inability of TZDs to induce few PPARα-responsive genes, such as the carnitine palmitoyltransferase gene (Cpt-I), suggests that they may require the presence of other coactivators or may be under dominant regulatory control of other transcription factors. However, DeLuca et al. [25] demonstrated that TZDs induce acetyl CoA oxidase (Aco) and fatty acid binding protein 1 (Fabp1), which are known as PPARα target genes in both wild type and PPARα null mice, without any increase of PPARγ expression. In addition, it should be noted that Brun et al. [26] have observed some degree of cross-activation between PPARγ and PPARα with respect to the transcription of adipocyte differentiation genes, suggesting that residual PPARγ receptor expressed in liver may be sufficient to mimicking PPARα function.

Table 1: A literature survey of gene expression changes after treatment of *in vivo* and *in vitro* rodent liver models with PPARγ agonists.

		IN VIVO												*IN VITRO*						
	Ref.	[27]	[28]	[29]			[24]	[30]	[11]	[31]		[23]	[32]	[33]	[34]	[12]	[35]	[36]	[36]	[37]
	model	PPARα$^{-/-}$ mice PPARγ1 overexpression		AZIP	WT LKO	AZIP LKO	ob/ob	ZDF rats	WT mice AZIP	WT	ap2/dta	sprague dawley rats	KKA ob/ob	primary rat hepatocytes					c9 rat	aml-12 murine
	treatment	TRO 5 d 0.1% v/v	no treatment	ROSI 5 w 5 mg/kg/d			TRO 10 d 200 mg/100 g chow	GW 1929 7 d 5 mg/kg/d	ROSI 3 w 3 mg/kg/d	TRO 5 w 16.6 mg/g chow		ROSI 4 d 30 mg/kg/d	ROSI 28 d 2.5 mg/kg/d and TRO 100 mg/kg/d d	TRO 6 h 10–200 μM	TRO 24 h 5–50–200 μM	TRO, ROSI, PIO, CIG 6 h–10 h 5–200 μM	TRO 24 h 25 μM	TRO, ROSI, PIO 24 h 10–50–100 μM	TRO, ROSI, PIO, CIG 6 h–10 h 5–200 μM	TRO 5 d 5 μM
	method	affymetrix/q-PCR		northern blotting				northern blotting/ q-PCR		northern blotting			q-PCR	northern blotting	q-PCR	applied biosystem	q-PCR	q-PCR/eXpress		q-PCR
Oxidative stress	Por																	+		
	Hmox1															+		+	+	
	Ephx2						−													
	Nqo																	+	+	
	Acox1			+	−	+														
	Cox2																		+	
	Serpine1	−																		
Cell cycle, proliferation and death	Kif5b	+																		
	Brca1	+																		
	Cdkn1c	+																		
	Gadd45a	−																		
	Kif24	+																		
	Gadd153															+			+	
	Atf3															+				
	Bcl2l11															+				
	Blnk															+				
	p21	+																	+	
	Gadd45g															+		+	+	
	Cflar															+				
	Egr1															+				
	Emp2															+				
	Fem1b															+				
	Fhl2															+				
	Fos															+				
	Herpud1															+				
	Hmga1															+				
	Igfbp1															+				
	Igfbp6															+				
	Il7															+				
	Lats1															+				
	Mt1a															+				
	Nr1d1															+				
	Phlda1															+				
	Sfn															+				
	Skil															+				
	Stk17b															+				
	Bid3															+				
	Ckn1b															+				
	Cldnk4															+				
	Myd116															+				
	Trp53inp1															+				
	S100a10		+																	
	Kif5b	+																		
	Il7	−																		
	Actn1														+					
	Cdh1														−					
	Gsn														−					
	Ucp2	+					+													

Table 1: Continued.

		IN VIVO														IN VITRO						
	Ref.	[27]	[28]	[29]			[24]	[30]		[11]	[31]		[23]	[32]		[33]	[34]	[12]	[35]	[36]	[36]	[37]
	model	PPAR$\alpha^{-/-}$ mice PPARγ1 overexpression		AZIP	WT LKO	AZIP LKO	ob/ob	ZDF rats	WT mice	AZIP	WT	ap2/dta	sprague dawley rats	KKA	ob/ob	primary rat hepatocytes					c9 rat	aml-12 murine
	treatment	TRO 5 d 0.1% v/v	no treatment	ROSI 5 w 5 mg/kg/d			TRO 10 d 200 mg/100 g chow	GW 1929 7 d 5 mg/kg/d	ROSI 3 w 3 mg/kg/d		TRO 5 w 16.6 mg/g chow		ROSI 4 d 30 mg/kg/d	ROSI 28 d 2.5 mg/kg/d and TRO 100 mg/kg/d d		TRO 6 h 10–200 μM	TRO 24 h 5–50–200 μM	TRO, ROSI, PIO, CIG 6 h–10 h 5–200 μM	TRO 24 h 25 μM	TRO, ROSI, PIO 24 h 10–50–100 μM	TRO, ROSI, PIO, CIG 6 h–10 h 5–200 μM	TRO 5 d 5 μM
	method	affymetrix/q-PCR		northern blotting				northern blotting/q-PCR			northern blotting			q-PCR		northern blotting	q-PCR	applied biosystem	q-PCR	q-PCR/eXpress		q-PCR
Lipid metabolism	Fsp27	+	+																			
	Acaca			+	–	+																
	Lipe		+																			
	Srebp1							0	0													+
	Fabp4	+	+				+			+												+
	Adfp			+	–	+																
	Cd36	+	+	+	–	+	+															
	Cpt1a			+	–	–	0		0	0												
	Cpt1b			+	–	–	0		0	0												
	Pex11a	+																				
	Acsl5							+														
	Apoc3						0															
	Dci							–														
	Elovl4	+																				
	Fabp5	+																				
	Fasn			+	–	+		+		–												+
	Ldlr			+	+	–																
	Lipc			+	+	+																
	Acdl							–														
	Thiolase		+					–														
	Kiaa1881	+	+																			
	Lamb3		+																			
	Me1							+					+									
	Mttp			–	–	–																
	Peci							–														
	Pnpla2	+																				
	Scd1			+	–	+				+												
Xenobiotic metabolism	Cyp1a1																		0			
	Cyp1a2	–																	0			
	Cyp2b1																		+	+		
	Cyp2b2																		+			
	Cyp2b6																					+
	Cyp3a																		+			
	Cyp3a1																			+		+
	Cyp3a3																+					
	Cyp4b1		+																			
	Cyp4a10	+	+										+									
	Cyp4a14	+	+																			
	Cyp51a1							+														
	Gsta2	–	–																			
Carbohydrate metabolism	G6pc							–														
	Pdk4	+																				
	Pepck							–														
	Gk							+														
	Irs2								–													
	Pc	+	+					–														
	Aqp7	+																				

Table 1: Continued.

		IN VIVO													*IN VITRO*						
	Ref.	[27]	[28]	[29]			[24]	[30]		[11]	[31]		[23]	[32]		[33]	[34]	[12]	[35]	[36]	[36]	[37]
	model	PPARα$^{-/-}$ PPARγ1 overexpression	mice	AZIP	WT LKO	AZIP LKO	ob/ob	ZDF rats	WT mice	AZIP	WT	ap2/dta	sprague dawley rats	KKA	ob/ob	primary rat hepatocytes					c9 rat	aml-12 murine
	treatment	TRO 5 d 0.1% v/v	no treatment	ROSI 5 w 5 mg/kg/d			TRO 10 d 200 mg/100 g chow	GW 1929 7 d 5 mg/kg/d	ROSI 3 w 3 mg/kg/d		TRO 5 w 16.6 mg/g chow		ROSI 4 d 30 mg/kg/d	ROSI 28 d 2.5 mg/kg/d and TRO 100 mg/kg/d d		TRO 6 h 10–200 μM	TRO 24 h 5–50–200 μM	TRO, ROSI, PIO, CIG 6 h–10 h 5–200 μM	TRO 24 h 25 μM	TRO, ROSI, PIO 24 h 10–50–100 μM	TRO, ROSI, PIO, CIG 6 h–10 h 5–200 μM	TRO 5 d 5 μM
	method	affymetrix/q-PCR		northern blotting				northern blotting/q-PCR			northern blotting			q-PCR		northern blotting	q-PCR	applied biosystem	q-PCR	q-PCR/eXpress		q-PCR
Transporters	Abcb1		+																			
	Abcc3:																+					
	Slco1b1	+																				
	Slco1b3																−					
	Mdr2																+					
	Slco4a1	+																				
	Slc25a1							+														
Nuclear receptors	Nr1i3																+					
	Hnf4a																−					−
	Ppara			+	−	+			0	0												
	Pparg		+	+	−	+				+	−	+				+		+				
	Cebpa																−					
	Pparg1													+	+							
	Pparg2														+							
	Ces3		−																			
	Hspa1																	+				
	Il6r																	+				
	Vnn1	+																				
	Scd2																+					
	Actg1							+														
	Adipoq	+	+																			
	Cav1	+	+																			
	Cav2	+																				
	Cfd	+	+																			
Miscellanous	Lrp1			+	+	+																
	Mapk		+																			
	Paqr7	+																				
	Tmem159	+																				
	Tpm2	+																				
	Aco						+															
	Aox			+	+	+			+	+												
	growth response protein												+									
	Fdft1							+														
	Hex												+									
	Hmgcs2							−														
	Igh-6							+														
	Il8bp		−																			
	Ldh-a												+									

+: up-regulated
−: down-regulated
0: not modulated
The case is empty when the gene has not been studied.

Although preclinical animal studies have not allowed the prediction of glitazone hepatotoxicity in humans, several studies in animals have dealt with the mechanisms of TZDs hepatotoxicity. A role of Cyp 3a1 has been advanced for the enhanced acetaminophen toxicity in rats when this compound is administered with TRO [38]. However, chemical inhibition of drug metabolizing enzymes involved in TRO metabolism did not protect against TRO-induced toxicity. Another mechanism of toxicity could be the inhibition of the activity of the bile salt export pump (BSEP or ABCB11), which is responsible for cholestasis [39]. However, TRO acts largely through the induction of apoptosis and the more likely mechanism is via effects on mitochondria resulting in the depletion of ATP and the release of cytochrome c [40]. Interestingly, it has been recently shown that in a specific mouse model $Sod2^{+/-}$ (whose mitochondrial antioxidant defense is slightly compromised), low repeated doses of TRO resulted in an oxidant injury to liver mitochondria, giving further support to mitochondria as targets for TRO-induced liver injury [41]. Nevertheless, any of the effects could explain the initiating mechanism. TZDs also have anti-inflammatory properties. They inhibit macrophage activation and down-regulate proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in the liver of liposaccharide-stimulated mice [42, 43].

2.2. Effects of PPARγ Agonists in Obese and Diabetic Liver. Several studies have dealt with the effects of PPARγ agonists in obese and diabetic mouse liver. Enhanced levels of PPARγ have been observed in the fatty liver of several animal models of obesity and diabetes, including ob/ob, db/db, A-Zip, and KKAγ mice [24, 29, 32]. Thus, KKAγ and ob/ob mice exhibit 8- and 6-fold more hepatic PPARγ transcripts than C57BL/6 mice [32]. This increase was more pronounced compared to that of PPARα mRNAs. On the contrary, Burant et al. [31] showed a 25% reduction in PPARγ mRNA levels after TRO treatment of wild type animals.

The mechanistic relationship between steatosis and the increase of PPARγ expression in the liver is still unclear. It is possible that elevated PPARγ expression in ob/ob livers appears to be a pathophysiological response to the severity state of obesity and diabetes. In this regard, transcriptional effects of TZDs on their target genes have been shown to be exacerbated in obese diabetic versus lean control animals. Indeed, some genes were overexpressed in obese and diabetic mice compared to controls after treatment with TRO; for example, adipocyte fatty acid binding protein (Ap2 or Fabp4) and fatty acid translocase (Fat or Cd36) and others were increased only in the liver of ob/ob mice, for example, the uncoupling protein 2 gene (Ucp2) [24]. The fact that PPARγ target genes such as Cd36 were also induced by TRO treatment in lean mice without induction of PPARγ expression [24] suggests that glitazone effects in obese or diabetic rodent models are different from those occurring in lean control animals. Therefore, it is essential to estimate glitazone effects in regard to the metabolic status of the animals. Noteworthy, the genes found to be modulated in the liver after glitazone treatment were unchanged in the adipose tissue [24].

Some differences have been observed among glitazone effects. Thus, only ROSI was found to induce an increase of both liver weight and hepatic triglycerides in AZIP/F1 mice. This could be explained by PPARγ-independent mechanism effects [11]. However, another explanation is the higher affinity of ROSI than TRO for the PPARγ receptor. Indeed, ROSI caused higher incidence and severity of microvesicular steatosis in obese KKAγ mice compared to TRO due to its higher receptor affinity (approximately 100-fold) and its higher transcriptional response. In that study, the hepatic triglycerides content of treated and untreated animals was not different, leading to the conclusion that this microvesicular steatosis is not due to triglyceride accumulation. Exacerbation of fatty liver has also been reported with ROSI which exerted its effects on serum glucose levels independently of hepatic PPARγ levels [44]. Compared to wild type ob/ob mice, triglyceride content as well as mRNA levels of lipogenic genes, such as fatty acid synthase (Fasn), acetyl CoA carboxylase (Acc), and stearoyl CoA desaturase 1 (Scd1), were strongly decreased in corresponding PPARγ-deficient animals [44]. These data indicate that obese mice are more sensitive to the steatogenic effects of glitazones than lean animals.

It has been shown that a few PPARα target genes, such as Aco which is involved in peroxisomal β-oxidation, were deregulated in diabetic rodent models after TZDs treatment. Since PPARα and PPARγ recognize similar DNA response elements, it is quite conceivable that TZDs could modulate PPARα responsive genes in liver of obese mice [45]. In wild type rodents, TRO and ROSI cause a decrease in serum cholesterol, triglycerides, free fatty acid content and, obviously, glucose levels without modifying liver or body weight [11, 23, 31]. Their effects on these biological parameters are substantially higher in obese mice.

To understand the effects of increased PPARγ expression in fatty liver cells, Yu et al. used $PPAR\alpha^{-/-}$ mice and explored gene effects of PPARγ1 overexpression [27, 28]. Expression of genes involved in adipocyte differentiation and lipid metabolism was modulated in the liver of this KO mouse model. Noticeable increase was observed in Cd36, glucokinase (Gk), malic enzyme (Me), low-density lipoprotein (Ldl), microsomal transfer protein (Mtp), and angiopoietin-like 4 (Angptl4) in the absence of any change in CCAAT/enhancer binding protein alpha (C/ebpα), sterol regulatory element binding protein 1 (Srebp1), phosphoenolpyruvate carboxykinase (Pepck), and glucose transporter type 2 (Glut-2 or Slc2a2) expression levels, leading to the conclusion that there is an adipogenic conversion of the liver when PPARγ1 is overexpressed in this organ. Moreover, Vidal-Puig and coworkers demonstrated that forced expression of PPARγ2 or γ1 in fibroblasts was sufficient to drive the determination of an adipocyte cellular lineage [46]. Furthermore, the relative abundance of PPARα in normal liver might serve as a key regulator of fatty acid catabolism, thereby minimizing the need for pathological adipogenic transformation of hepatocytes to store lipids. PPARα and fatty acid oxidation activity might partially protect from too high PPARγ1

adipogenic activity in the liver. Way et al. analyzed transcript levels of genes involved in lipid and glucose homeostasis in Zucker Diabetic Fatty (ZDF) rats and concluded that PPARγ activation had coordinate effects on genes involved in important hepatic metabolic pathways such as Pepck and glucose 6 phosphatase (G6P) which were decreased [30].

2.3. Extrahepatic Effects of PPARγ Agonists. Marked tissue-differences are observed in the response to glitazones: relative to the liver and the skeletal muscle, PPARγ is 10- to 30-fold higher expressed in human and rodent adipose tissues [47]. Likewise, while PPARγ agonists affect only a small number of genes in the liver and the skeletal muscle, they cause conspicuous changes in gene expression in adipose tissues [30]. Thus, following a 14-day treatment of ZDF rats, ROSI decreased Tnf-α and increased glucose transporter 4 (Glut4), muscle carnitine palmitoyltransferase (Cat), stearoyl CoA desaturase (SCoA), and fatty acid translocase (Fat) in adipose tissue, while only Fat was slightly augmented in the liver which expresses very little hepatic PPARγ. Comparison with the effects of retinoid X receptor-selective agonists, such as LG100268, that also produce insulin sensitization in diabetic rats, showed that these agonists modulated different gene patterns from those observed with ROSI, indicating that these compounds may act by independent and tissue-specific mechanisms [48].

Similar tissue-differences were observed in diabetic (db/db) mice treated with PIO for 2 weeks. Analysis of 42 genes associated with diabetes by RT q-PCR showed that in the liver, expression of Gk, Glut-2, apolipoprotein A-IV (ApoA-IV), PPARγ, and a series of fatty acid oxidation enzymes were increased while those of triglyceride lipase, lipoprotein lipase, apolipoprotein A-I (Apo-AI), and insulin receptor substrate 2 (Irs-2) were decreased [49].

Glitazones decrease glucose concentrations not only by their action on adipocytes but also by their effects on the liver and muscle. Indeed in aP2/DTA mice, whose white and brown fat is virtually eliminated by fat-specific expression of diphterin toxin A chain, TRO alleviated hyperglycemia without affecting PPARγ levels in liver, suggesting independence from both adipose tissue and PPARγ receptor [31]. However, conflicting observations have been reported. Thus, after mouse treatment with PIO, gene expression of Pepck was found to be increased in the liver by Hofmann et al. [50] but only in muscle by Suzuki et al. [49]. Accordingly, increased expression of PPARγ in the liver of diabetic mice has been reported in certain studies [46].

PPARγ1 or γ2 mRNA levels are not affected in adipose tissue by obesity in the ob/ob and Gold ThioGlucose (GTG) animal models. Accordingly, Auboeuf et al. [51] demonstrated that obesity and non-insulin-dependent diabetes mellitus are not associated with alteration in PPARγ gene expression in adipose tissue in humans. However, conflicting observations have been made. Indeed, Vidal-Puig et al. [46] showed that expression of PPARγ2 mRNA is increased in adipose tissue of obese men and women, and that the ratio of PPARγ2/γ1 is directly correlated with their body mass index. In addition, they did not observe similar changes in muscle.

Besides its well-known function in adipocyte differentiation, PPARγ activation by TZDs leads to an anti-inflammatory response in adipose tissue. This has been observed in fat deposits of various obese or diabetic rodent models [52] and in fat biopsies of type 2 diabetic patients [53]. This anti-inflammatory response can be assessed by the inhibition of expression and/or biological activity of several proinflammatory factors such as TNFα, IL-6, plasminogen activator inhibitor 1 (PAI-1), monocyte chemoattractant protein 1 (MCP-1), and angiotensinogen [54]. Proposed molecular mechanisms, underlying this effect, include inhibition of the intracellular NF-kappaB pathway [55] and activation of nuclear translocation of the glucocorticoid receptor [56].

Macrophages accumulate in adipose tissue of obese animals, where they can produce inflammatory mediators, contributing by this way to insulin resistance [57]. Targeted deletion of PPARγ in macrophages severely impaired TZDs response in mice submitted to a high fat diet [58]. These data emphasize the crucial role of macrophages for obtaining full effects of TZDs in the context of insulin resistance or in diabetic conditions.

Despite its weak expression level, PPARγ is thought to play a role as a regulator of insulin action in the skeletal muscle [59]. Indeed, it has recently been shown that muscle-specific PPARγ deletion in mouse caused insulin resistance [60]. PIO treatment of a murine model of myoblasts, the C2C12 cells, improved insulin sensitivity as assessed by increased glucose uptake [61]. Moreover, some data indicate that PPARγ activation in skeletal muscle could contribute to the beneficial effect of TZDs. Indeed, experiments on myocyte models have shown that ROSI induced local expression of the insulin-sensitizing hormone adiponectin [62]. However, conflicting results on the role of PPARγ in muscle have also been published, showing that muscle specific PPARγ KO did not impair TZD action in a mouse model of insulin resistance [63]. These results raise the question of which tissues are really necessary to achieve pharmacological action of TZDs. A precise analysis of PPARγ regulation in other tissues where its expression reaches a sufficient level could lead to an answer.

3. *In Vitro* Animal Studies

Cytotoxicity studies have shown that primary rat hepatocytes were not more sensitive to TZDs than cells that did not express the drug metabolizing enzymes involved in their metabolism. TRO was more toxic than ROSI and PIO at equimolar concentrations [12]. TRO induced a decline in mitochondrial transmembrane potential and apoptosis as well as an oxidative stress. These effects were also observed in other cell types and on isolated mitochondria [64].

Several studies have been carried out on modulation of gene expression by TZDs in rodent hepatocytes (Table 1).

Different concentrations and exposure times have been tested although a 24h-treatment was the most frequent. Using Applied Biosystem rat genome survey microarrays with 26857 probes, Guo et al. [12] compared the effects of five PPARγ agonists, including TRO, CIG, ROSI, and PIO, in rat hepatocytes after a 6-hour treatment. Around 2-fold more genes were modulated in TRO- and CIG- than in ROSI- and PIO-treated cell samples. Genes related to cell death were deregulated only with the most cytotoxic TRO and CIG concentrations. Similar observations were reported by Vansant et al. [36]. TRO was also found to modulate more genes than other glitazones (ROSI, PIO) at the same concentration, especially genes related to oxidative stress, DNA repair, and cell death, such as heme oxygenase 1 (Ho-1), NAD(P)H quinone oxidoreductase (Nqo), growth arrest DNA-damage-inducible 45 (Gadd45), FBJ osteosarcoma oncogene (Fos), BCL2-like 11 (Bcl2l11), and BH3 interacting domain 3 (Bid3). A TRO response closer to CIG than to the second TZDs generation, ROSI and PIO, was also found in the C9 rat liver cell line [36].

As observed *in vivo* [31], TRO induced expression of the PPARγ gene [33] and repressed genes related to lipid metabolism, such as Fasn and Cebp/α, in cultured rat hepatocytes [34]. Cyp induction by TZDs was evidenced in cultured primary rat hepatocytes using RTq-PCR analyses. Thus Cyp 3a and 2b subfamily genes were increased after exposure to TRO, ROSI and PIO [35, 36, 65]. Other genes including multidrug resistance (Mdr) 2 and 3, cadherin and superoxide dismutase (Sod) 2, were also up-regulated while Mdr1 and organic anion transporting polypeptide 8 (Oatp 8) were down-regulated [34].

PPARγ2 expression was shown to induce lipid accumulation in the mouse AML12 liver cell line stably expressing PPARγ2, and several genes known to be overexpressed in steatotic liver of ob/ob mice were found to be up-regulated by TRO, such as adipose differentiation-related protein (Adrp), Fabp4, Srebp1, Fasn, and Acc by q-PCR analysis. Lipid accumulation and the lipid droplet protein were further increased after a 7-day treatment with TRO [37].

An extensive study of gene expression changes induced by TZDs has also been performed on the mouse 3T3-L1 adipocyte cells using microarrays and RTq-PCR [9]. Expression gene profiles obtained with TRO, ROSI and PIO tested at concentrations that elicited maximum biological effects (i.e., 20 μM for PIO and TRO and 1 μM for ROSI) were distinct but with an overlapping: 94 out of the 326 deregulated genes were found to be modulated by the three glitazones after a 24-hour treatment. For example, pepck, pyruvate dehydrogenase kinase 4 (Pdk4) and c-Cbl-associated protein (Cap) were activated by the three compounds but with different time-curves, suggesting different mechanisms of gene regulation. Moreover, ROSI and PIO were more potent than TRO in activating Pepck and Pdk4 and repressing regulator of G-protein signaling 2 (Rgs2). These data support the conclusion that gene profile changes induced by TZDs are different in liver cells and in adipocytes, in agreement with *in vivo* observations.

4. *In Vitro* Human Liver Cell Studies

Most studies on the effects of TZDs in human liver have been performed with primary hepatocyte cultures or hepatoma cell lines. Primary human hepatocytes are recognized as the most pertinent *in vitro* model but they exhibit early phenotypic alterations and their survival does not exceed a few days in standard culture conditions. Human hepatocytes have, in addition, a scarce and unpredictable availability and are characterized by large interdonor functional variability [66]. Hepatic cell lines were thought to be an alternative but most of them have lost most of, if not all, their bioactivation capacity and consequently are of limited interest. In this regard, the new human hepatoma HepaRG cell line seems as an exception [67]. HepaRG cells exhibit a capacity of trans-differentiation; they undergo morphological and functional features of liver bipotent progenitor cells after plating and at subconfluence, lose expression of progenitor markers and differentiate into either hepatocyte-like or cholangiocyte-like cells [68]. Differentiated HepaRG cells possess most functional activities of primary mature hepatocytes and the indefinite growth capacity of hepatoma cells [69]. Noteworthy, they express major cytochromes P450, conjugating enzymes, and plasma transporters [70].

The mechanism(s) of TZD hepatotoxicity in humans still remain(s) controversial. Several proposals have been advanced to explain the induction of apoptosis by TRO namely accumulation of toxic metabolites or bile acids, mitochondrial damage, and oxidative stress. TRO has been shown to be metabolized by CYP3A4 (the homolog of Cyp3a1 in rodent) to a very active quinone metabolite which is able to produce reactive oxygen species via the redox/cycling or to bind to cellular proteins [40]. This CYP3A4-mediated metabolism is in accordance with the frequent occurrence of centrilobular necrosis of the liver. CYP3A4 is also induced by TRO in primary human hepatocytes [71, 72] and a correlation has been observed between CYP3A4 levels and hepatocyte sensitivity to glitazone [73].

Studies dealing with the effects of TZDs on gene expression in either primary human hepatocyte cultures or human hepatoma cell lines are scarce and most of them studied only few genes [76, 79] (Table 2). Human hepatocytes were slightly more sensitive than their rat counterparts [34], but less sensitive than human hepatoma HepG2 cells to cytotoxicity induced by TRO, supporting the conclusion of an absence of correlation between TRO toxicity and its hepatic metabolism. TRO was found to induce cell arrest and to cause time- and concentration-dependent apoptosis in various liver cell types [75, 77, 83]. Cell arrest was associated with increased expression of a cascade of cyclin-dependent kinase inhibitors, that is, cdki p21, p27, and p18 that each plays a crucial role in adipocyte differentiation through PPARγ activation [81]. This increase occurred through down-regulation of nuclear S-phase kinase-associated protein 2 (SKP2) [80]. Apoptosis was associated with activation of both c-Jun N-terminal protein kinase and p38 kinase and overexpression of proapoptotic proteins and cyclooxygenase 2 (COX-2) [77, 83]. These effects are not limited to hepatoma cell lines. Indeed, TRO also induced growth arrest of prostate

Table 2: Modulation of gene expression in human liver cell models after treatment with PPARγ agonists.

	Ref.	[34]	[35]	[65]	Rogue et al. unpublished															[74]	[75]	[76]	[77]	[78]	[79]	[80]	[81]
	model	PHH			PHH DONOR 1		PHH DONOR 2		PHH DONOR 3		PHH DONOR 4			PHH DONOR 5			HepaRG cells			HepaRG cells	Hep3b Huh7	Huh7	Hepg2			HLF	HLF, HAK, HuH-7
	treatment	TRO 24 h 5,50,100 μM-	TRO 24 h 25 μM-	TRO 24 h 10 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 40 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 40 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 40 μM-	TRO 24 h 0,024 μM–25 μM	TRO04 8 h - 50 μM-	TRO-ROSI 1–8–24 h 50 μM-	TRO 6 h 30 μM-	TRO-4 h 10–30 μ M	TRO-ROSI 48 h 25–100 μM-	TRO for up 48 h 50 μM	TRO 24 h 50 μM
	method	q-PCR		Amersham	Agilent												Agilent			q-PCR		Super Array Bioscience qPCR	q-PCR/northern blotting	q-PCR			
Transporters	ABCB1	+			0	+	0	0	+	+	0	0	+	+	+	+	0	0	0								
	ABCC2	+			0	0	0	0	0	0	0	0	0	0	0	+	0	0	0								
	ABCC3	+			+	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	SLC10A1	−			0	0	0	0	0	0	0	0	0	0	0	−	−	−	−								
	ABCB4	+			0	0	0	0	+	+	0	+	+	+	+	+	0	0	0								
	SLCO1B1	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	−								
	SLCO1B3	−			−	−	−	−	−	−	0	−	−	0	−	−	0	0	0								
Cell cycle, proliferation, death and differentiation	CCND1	+			0	+	+	+	0	+	0	0	0	0	0	0	0	0	0								
	CDKN1A	0			0	0	+	+	+	+	0	0	0	0	0	0	0	0	0							+	+
	GADD45G	+			0	+	0	0	0	+	0	0	0	+	+	+	0	0	0								
	AFP	−			0	−	0	−	0	0	0	−	−	0	0	0	0	0	0								
	TGFA	+			0	0	0	−	−	−	0	0	0	0	0	0	0	0	0								
	CCNE1				0	0	0	0	0	0	0	0	0	0	0	0	0	0	+							0	0
	ALB	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	−								
	CDKN1B				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0							0	0
	JUND	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	CCNG1	0			0	0	−	−	0	−	0	0	0	0	0	0	0	0	0								
	MYC	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	TGFB1	0			0	0	+	+	0	+	0	0	0	0	0	0	0	0	0								
	ALPL	0			0	0	0	0	0	0	0	0	+	0	0	0	0	0	−								
	GADD45A	+			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	IGFBP1				0	0	0	−	−	−	0	0	0	0	+	0	0	0	+						+		
	SKP2				0	0	0	0	0	0	0	0	0	0	0	0	0	0	+							−	
Lipid metabolism	FABP1	+			+	+	+	+	+	+	0	+	+	+	+	+	+	0	−								
	FASN	+			0	0	0	0	0	0	0	0	0	+	+	0	0	0	0								
	CPT1A	+			0	+	+	+	0	0	0	+	+	+	+	+	0	0	0								
	SREBF2				+	0	0	+	0	0	0	0	0	0	0	0	0	0	0					−			
	HMGCR				0	0	0	0	0	0	0	0	0	0	0	0	0	0	−					−			
	INSIG1				0	0	0	0	0	0	0	0	0	+	+	+	0	0	0					nd			
	LDLR				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0					−			
	INSIG2				0	0	0	0	0	−	0	0	0	0	0	0	0	0	0					+			
Xenobiotic metabolism	CYP1A1		0		+	+	0	0	0	+	0	0	0	0	0	0	0	−	0	0							
	CYP1A2	+	0		0	0	−	0	0	0	0	0	+	0	0	+	0	0	0	0							
	CYP2B6			+	+	+	0	0	+	+	0	+	+	0	0	+	0	0	0	+							
	CYP2C9	+			0	0	0	0	0	0	0	0	+	+	+	+	+	0	0								
	CYP2E1	+			0	0	−	−	−	−	0	0	0	0	0	0	0	0	−								
	CYP3A4	+	+		0	0	0	0	+	+	0	+	+	+	+	+	+	+	+	+							
	UGT1A10	+			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	GSTP1	+			0	0	0	0	−	−	0	0	0	0	0	0	0	0	0								
	GSTA1	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	−								

Table 2: Continued.

	Ref.	[34]	[35]	[65]	Rogue et al. unpublished															[74]	[75]	[76]	[77]	[78]	[79]	[80]	[81]
	model	PHH			PHH DONOR 1		PHH DONOR 2		PHH DONOR 3		PHH DONOR 4			PHH DONOR 5			HepaRG cells			HepaRG cells	Hep3b Huh7	Huh7	Hepg2			HLF	HLF, HAK, HuH-7
	treatment	TRO 24 h 5,50,100 μM-	TRO 24 h 25 μM-	TRO 24 h 10 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 40 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 40 μM-	TRO 24 h 5 μM-	TRO 24 h 20 μM-	TRO 24 h 40 μM-	TRO 24 h 0,024 μM–25 μM	TRO04 8 h - 50 μM-	TRO-ROSI 1–8–24 h 50 μM-	TRO 6 h 30 μM-	TRO-4 h 10–30 μ M	TRO-ROSI 48 h 25–100 μM-	TRO for up 48 h 50 μM	TRO 24 h 50 μM
	method	q-PCR		Amersham	Agilent									Agilent						q-PCR		Super Array Bioscience qPCR	q-PCR/northern blotting	q-PCR			
Carbohydrate metabolism	G6PC				0	0	0	0	−	−	0	0	0	0	0	0	0	−	−								
	PDK4				−	0	−	−	−	−	0	+	+	+	+	+	0	+	+								
	PEPCK				0	0	0	0	−	−	0	0	0	0	0	+	+	+	+								
	FBP1	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	−								
Oxidative stres	HMOX1	+			+	+	+	+	+	+	+	+	+	0	0	0	0	+	+								
	PTGS2				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		−		−				
	HSPA1A	+			+	+	0	0	+	+	0	0	0	0	0	0	0	0	0								
	TXN	+			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	COX-2																				−						
	CAT				−	0	0	−	−	0	0	0	0	0	0	0	0	0	0			+					
Transcription factors	HNF4A	+			+	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	PPARG				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		+			0			
	CEBPA	0			0	+	+	+	+	+	0	0	0	0	0	0	0	0	0								
	CEBPB	0			0	+	0	0	0	0	0	0	0	0	0	0	0	0	0								
	NR1I2	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	NR1I3	0			0	0	0	−	0	0	0	0	0	0	0	0	0	0	−								
Fbrosis/ senescence	GSN	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	TIMP1	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
	CDH1	+			+	+	0	0	0	0	0	0	0	0	0	0	−	0	0								
	RGN	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	−								
Miscellaneous	PDIA4				0	0	−	−	0	0	0	0	0	0	0	0	0	0	0						+		
	ACTA1	0			+	0	+	+	0	0	0	0	0	0	0	−	0	0	0								

+: up-regulated
−: down-regulated
0: not modulated
The case is empty when the gene has not been studied.
Differentiated HepaRG cells from three different passages and 2-day human hepatocyte cultures from 5 donors were treated for 24 h with different concentrations of TRO. 500 ng of RNA samples from control and treated cultures were separately reverse transcribed and amplified using Quick Amplification Labeling Kit (Agilent). Then they were hybridized using 4×44 K Agilent microarrays satisfying Minimum Information About a Microarray Experiment (MIAME) requirements as previously described [82]. Normalization algorithms and background subtractions were automatically applied to each array to reduce systematic errors and to adjust effects due to technological rather than biological variations using FE and Resolver softwares. The combination of technical and biological replicates uses the error-weighted log ratio average and an estimated error method of the Rosetta Resolver system.

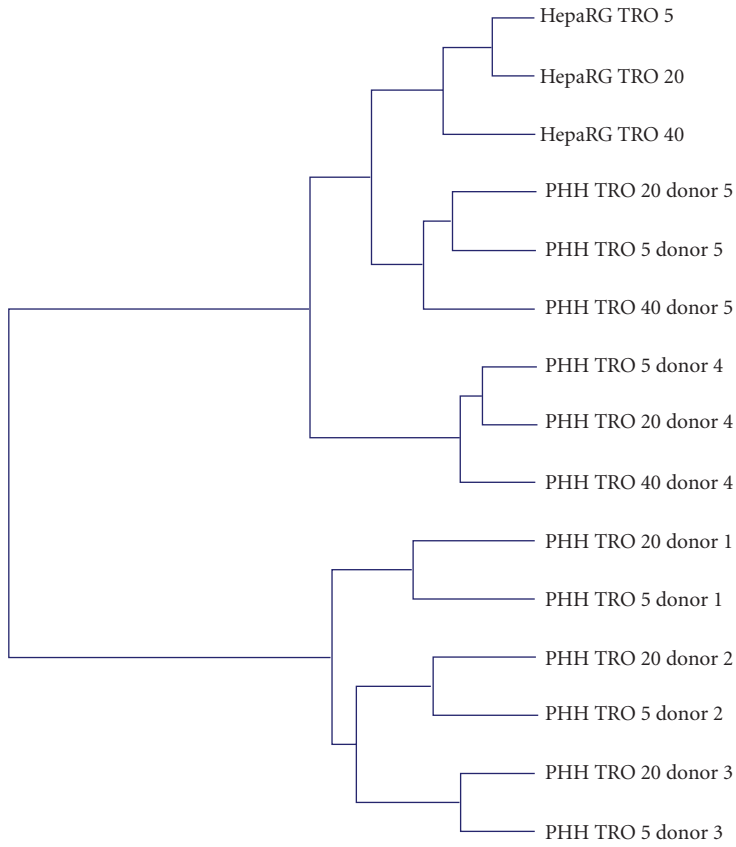

Figure 1: Two-dimensional hierarchical clustering of gene expression profiles induced by TRO treatment in primary human hepatocytes from five donors and HepaRG cells. The clustering was generated by using the Resolver system software with an agglomerative algorithm, the Ward's min variance link heuristic criteria, and the Euclidean distance metric. Cultures and microarray analysis as in Table 2.

and bladder carcinoma cell lines [84]. By contrast, other members of the glitazone family, ROSI or PIO, had no effect on the growth of these cell lines [84] and did not cause any apoptosis of HepG2 cells [83]. The endogenous ligand 15-deoxy-prostaglandin J2 was also found to inhibit growth of prostate and bladder carcinoma cell lines by inducing apoptosis [84]. Since these effects are selective of the PPARγ ligand and the cell line, they can be interpreted as PPARγ-independent effects [84]. Apoptosis induced by TRO in human MCF-7 breast carcinoma cells has been associated with induction of GADD45 gene expression [85] while growth inhibition has been correlated to overexpression of another DNA damage gene, GADD153, in nonsmall lung carcinoma cells [86].

As observed in rat hepatocytes, Kier and coworkers [87] showed that TRO induced more genes than ROSI in human hepatocytes. This observation was based on analysis of gene expression profiles and did not include individual characterization of deregulated genes. Other studies have shown a down-regulation of SREBP-2, a gene encoding the sterol regulatory element-binding protein-2 that mediates cholesterol synthesis, as well as the two SREBP-2 target genes, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) and low density lipoprotein receptor (LDLR), in HepG2 cells exposed to 30 μM TRO for 4 h [78]. In agreement with *in vivo* human data, TZDs were also found to modulate CYP activities in human hepatocyte cultures. CYP3A4 and CYP2B6 were induced by TRO [34] and only CYP2B6 by ROSI and PIO [35] in primary human hepatocytes. Similar observations were made in the well-differentiated HepaRG cell line [74].

Up to now, studies on human hepatocytes have been limited to a few donors (one to three), and no interdonor variability has been considered. Since interdonor variability in response to chemical inducers or inhibitors is well established, we recently compared the effects of TRO in human hepatocyte cultures from five donors after a 24-hour treatment using pangenomic microarrays (Rogue et al., unpublished data). Two-dimensional hierarchical clustering of gene expression profiles showed that hepatocyte populations separated according to the donor and not to the TRO concentration (Figure 1). It exhibited two separate clusters: one with donors 4 and 5 and the second with donors 1, 2, and 3. The number of genes modulated by TRO greatly varied as a function of the donor and drug concentration. At 5 and 20 μM, TRO modulated 5754 and 7266 genes, respectively, in at least one donor but only 4 and 29 genes in the 5 donors, respectively (Figure 2). The small subset of common deregulated genes in hepatocyte cultures from several donors is in agreement with the findings

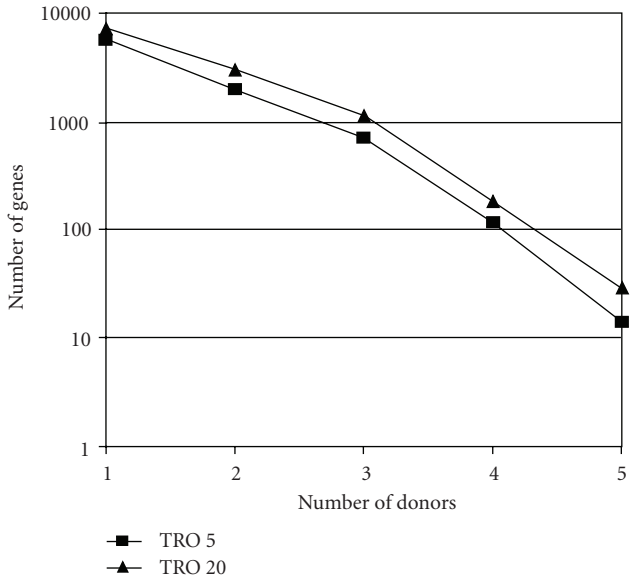

Figure 2: Total gene numbers modulated by 5 and 20 μM TRO in primary human hepatocytes from one to five donors (FC $\geq$ 1.5 pv $\leq$ 0.01). Cultures and microarray analysis as in Table 2.

reported by Goyak et al. [88], showing that the number of modulated genes deregulated in ten populations of human hepatocytes by arochlor 1254, di(2-ethylhexyl)phthalate, and phenobarbital did not exceed 0.1%. In our study, among the few genes deregulated in the five donors by 5 μM TRO, only two genes involved in oxidative stress, namely, mannose binding lectin 2 (MBL2) and serum/glucocorticoid regulated kinase 2 (SGK2), were induced. Genes involved in lipid metabolism, such as FABP1 were deregulated only by 20 μM TRO in all the donors. Several PPAR target genes, such as CYP4A1, CPT1, or CD36, were induced in the two hepatocyte cultures treated by 40 μM TRO.

Despite its therapeutic indications, TRO only slightly affected transcription of genes involved in glucose homeostasis. Fructose-1,6-bisphosphatase 1 (FBP1), an enzyme involved in gluconeogenesis, was not modulated in any of the five donors, in agreement with previous observations [34] while PDK4 and PEPCK were differently regulated across the donors. Their transcription could be either induced, repressed, or not affected by TRO treatment.

Comparison of gene profiles after TRO treatment in five human hepatocyte populations and the well-differentiated human hepatoma HepaRG cells evidenced a clear separation between the two cell models by two-dimensional hierarchical clustering. HepaRG cell samples separated as a function of TRO concentration and the dendrogram showed that they were closer to donors 4 and 5 than to donors 1, 2, and 3. The number of commonly modulated genes between HepaRG cells and primary human hepatocytes increased with the drug concentration; it was higher than the number of commonly modulated genes in four out of five donors. Among them, genes involved in lipid metabolism, such as FABP4 or CD36, were induced. Taken altogether our data support the view that the effects induced by TRO and more generally by PPARγ agonists are quite variable from one individual to another. This could explain the occurrence of toxic effects in only few treated patients. However, whether it would be possible to predict their potential hepatotoxicity in some patients on the basis of analysis of the expression level of a peculiar gene subset requires further studies.

5. Conclusions

Despite the numerous published studies on TZDs, their pharmacological and toxicological effects still remain obscure. Adipose tissue seems to be a predominant target organ. However, achievement of TZD pharmacological efficiency is obtained not only through an adipose-mediated mechanism but also requires an action in other organs, notably liver and skeletal muscle and also, as recently reported, in macrophages [58]. Moreover, PPARγ itself is required in a majority of metabolic tissues for regulation of insulin action and normal physiologic response to nutrients and it plays a critical role in the development of steatosis.

The determinants of susceptibility to glitazone-induced idiosyncratic hepatotoxicity remain to be elucidated. Studies have mainly concerned the most cytotoxic compound TRO. Evidence has been provided that toxicity is not directly related to its metabolism and the generation of a quinone metabolite. Direct toxicity caused by mitochondrial dysfunction has been demonstrated using both hepatic and nonhepatic *in vitro* models. Whether hepatotoxic effects of TZDs are related to PPARγ activation is not clear. PPARγ is only poorly expressed in the liver and both dependent and independent effects of glitazones have been seen.

Most *in vitro* studies have been performed with TRO concentrations of 50 μM or more while maximum plasma concentrations reached 3 to 6 μM in humans, making questionable the extrapolation of *in vitro* data to the *in vivo* situation. In comparison to TRO, ROSI is less toxic. The daily dose necessary for TRO therapeutic efficacy was 200 to 600 mg/day while it is only 4 to 8 mg/day for ROSI, indicating that patients were exposed to quite different doses between the first and second generations of TZDs [89].

During the last years, some studies have been designed to identify potential hepatic target genes in *in vivo* and *in vitro* models using RT-qPCR and microarray technologies. The amount of available data is still limited and has been obtained from different experimental conditions. However, some genes have been found to be usually modulated, being mainly related to drug and lipid metabolism. Interestingly, we have observed massive interindividual variability in the response of primary human hepatocytes to TRO treatment that could reflect the human situation. However, much more work is needed in order to identify the more pertinent genes which are differently expressed in the human hepatocyte populations and to determine whether the most critical effects in the liver are dependent or not on PPARγ activation. Moreover, it would be important to estimate the effects resulting from long-term repeated glitazone treatments and to determine if intracellular PPARγ levels in human liver cells are a critical parameter. The possible long-term treatments

of differentiated normal and steatotic human hepatoma HepaRG cells could represent a unique way to better understand hepatotoxicity of PPARγ agonists.

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